BIDMC Course on Comparative Physiology

Sunday 8/26/12 – Saturday 9/1/12

Mount Desert Island Biological Lab
Salisbury Cove
Maine

Introduction

About the course

This one week intensive course for medical residents at the Beth Israel Deaconess Medical Center is designed to engender an understanding of, and appreciation for major physiological mechanisms. The course will provide residents will an opportunity to conduct hands-on research using invertebrates and fish. Working in teams, they will generate, interpret and present data relevant to the hematologic and cardiovascular systems, as well as water homeostasis and salt secretion. Using a comparative approach, participants will gain an understanding of fundamental physiological processes that are common to humans.

About the venue

The Mount Desert Island Biological Laboratory (MDIBL) is an independent, nonprofit, marine research institution founded in 1898. The Laboratory is located in the village of Salisbury Cove, Maine, a small community wrapped around sheltered coves and wooded peninsulas on the northern edge of Mount Desert Island. Year round and seasonal research is conducted at the MDIBL in the areas of marine biomedicine and physiology, marine molecular biology and functional genomics, bioinformatics, environmental toxicology and toxicogenomics, transgenic species, and neuroscience. Hands-on educational programs provide research experience and training to scientists at all levels, from high school to senior investigators. The Maine IDeA Network for Biomedical Research--a research and education network linking MDIBL, Bates College, Bowdoin College, Colby College, College of the Atlantic, The Jackson Laboratory, The University of Maine, The University of Maine-Machias, and The University of Maine-Farmington--is based at MDIBL. MDIBL employs 37 scientists and staff year-round and has a vibrant summer community of scientists and students that can reach 200. Students and scientists live and work on campus. Eleven small laboratories perch over the water, and 28 cottages and dormitories are situated in the woods and around the coves that surround Laboratory Point. The social center of campus is the COOP, where everyone gathers for lunch and where students relax at the end of the day. Courses and conferences are held in Maren Auditorium and Dahlgren Hall--a new auditorium and restored historic schoolhouse paradoxically linked to form one conference center.

History of MDIBL

The Mount Desert Island Biological Laboratory is an independent, nonprofit, marine biomedical research institution located in Salisbury Cove, Maine, founded as the Tufts Summer School of Biology in 1898 at South Harpswell by Professor J.S. Kingsley of Tufts University.

The Laboratory was originally envisioned to be a summer school for undergraduates in biology. For seven or eight years, instruction was carried out along with research, but the instruction was dropped in favor of pure research. Early work was done on comparative anatomy and embryology of marine species and plants collected locally from the waters around South Harpswell, in southern Maine. The laboratory consisted of a single research building and a house occupied by Kingsley and his family. Visiting scientists and their families were frequently housed in tents during the summer research season. Research work continued at the South Harpswell site until 1921.

In June of 1921 the Laboratory was packed onto a boat and sailed Downeast to its present location at Salisbury Cove. The move was undertaken when an offer of land was made by an organization known as the Wild Gardens of Acadia—a land-holding group headed by George B. Dorr and John D. Rockefeller, Jr. instrumental in the founding of Acadia National Park.

A nearly identical laboratory was quickly built and research commenced again that summer. Seasonal research focused on the function of fish kidneys as a model for human kidney function. This signaled a shift from studying entire species to examining individual organs to determine their function within a living system. Work continued each summer until the lab was briefly closed during World War II. Following the war the lab resumed its summer schedule, focusing ever more deeply into cell structures and growth.

Starting in the early fifties there was a slow increase in the number of research personnel each year as more students were brought to the lab to assist with the research. By the early sixties the number of investigators had risen from 20 to 30 each summer to about 45. Researchers were now pursuing how cells regulated their functions and how various compounds affected cell operations.

Notable during this time was the appointment of MDIBL investigator James A. Shannon to head the National Institutes of Health as well as the work of E.K. Marshall and Homer Smith whose renal physiology studies led to understanding how the kidney and the gill regulated the body’s salt concentration of cells. With Robert Berliner and other investigators they studied the function of these organs.

In 1959, Wendell Burger’s discovery of the shark rectal gland’s function in pumping out salt from the intestine showed the shark gland to be an incredible model for the study of cystic fibrosis.

The sixties also brought about social changes which were reflected in some of the research done at the lab. The human race had now seen the planet from space and began to view the Earth as a finite resource that should be protected instead of exploited. Exploration into the effects of human activities showed how

damaging compounds such as DDT, crude oil, and other toxins were to the marine environment.

A major change in the laboratory occurred in 1971 with the first year-round investigations, led by Bodil Schmidt-Nielsen and William Kinter, who conducted research in kidney physiology. Most of the labs on campus were not built for winter occupation since they had no insulation or sources of heat, a state which a few labs are still in today. Research work was conducted in cell biology, fluid and ion transport, and cell metabolism.

In 1985, the NIEHS Center for Membrane Toxicity Studies (CMTS) was established at MDIBL. The CMTS was the first national research center established at MDIBL, and continues to conduct active studies in the areas of signal transduction and ion transport, and xenobiotic transport and excretion. Xenobiotics are foreign substances toxic to cells and cell function, including heavy metals such as arsenic, lead, tin, copper, zinc, and cadmium.

The last decade of the twentieth century introduced the field of molecular and cellular biology to the work done at the Laboratory. This has added a new dimension to the models used for research into marine species. In 1999, the Center for Functional Marine Genomic Studies was established and the Comparative Toxicogenomics Database was started in 2001.

That same year, MDIBL established a Biomedical Research Infrastructure Network (BRIN), a partnership between MDIBL, The Jackson Laboratory, Bates College, Colby College, Bowdoin College, College of the Atlantic and the University of Maine for biomedical research training in Maine. BRIN was later changed to the IDeA Network for Biomedical Research Excellence (INBRE).

In the fall of 2002, MDIBL launched its Marine Stem Cell Lines and Stem Cell Program, with David Barnes and Denry Sato, as Director and Deputy Director, respectively.

Today, there are 10 separate laboratory buildings with a total of 32 laboratory units. The year-round population has grown to 31 scientists and staff, with summertime growth up to 200 seasonal scientists, students, technicians and staff.

Source: http://www.mdibl.org/info/history.shtml

Course Directors

Mark Zeidel, M.D.
William C. Aird, M.D.

Core Faculty

Hematology
Marianne Grant, Ph.D. (Leader)
William Aird, M.D.
Ryan Nall M.D. (CMR)

Pharmacology of vascular smooth muscle
David Evans, Ph.D. (Leader)
Kelly Hyndman, Ph.D.
Mary LaSalvia, M.D. (ID fellow, MDI alum)

Secretory physiology
Patricio Silva, M.D. (Leader)
Robert Cohen, M.D.
Kate Spokes
Byron Vaughn, M.D. (GI fellow, MDI alum)

Salt and water homeostasis
Mark Zeidel, M.D. (Leader)
Warren Hill, Ph.D.
Bryce MacIver, Ph.D.
John Mathai, Ph.D.
Shani Herzig, M.D. M.P.H.

Cardiac augmentation
Richard Solomon, M.D. (Leader)
Federica del Monte, M.D., Ph.D.

Gastric Acid Secretion
Susan Hagen, Ph.D (Leader)
Carl Hauser, M.D.
Eric Cohen, M.D.
Hilary Womble, M.D. (CMR)

Meet the Faculty

William C. Aird, M.D. is Professor of Medicine at Harvard Medical School, Chief, Division of Molecular and Vascular Medicine and Director, Center for Vascular Biology Research at the BIDMC. Dr. Aird's major research interest is endothelial biology (he has recently published a 2000-page book on Endothelial Biomedicine with Cambridge University Press). Dr. Aird has a longstanding interest in comparative physiology, evolutionary biology and Darwinian Medicine.

Eric Cohen, M.D. is Instructor in Medicine at Harvard Medical School, and an Attending Physician at Beth Israel Deaconess Medical Center. Cohen received his medical training at the Sackler School of Medicine as part of the New York State American program in Tel Aviv, Israel. He has completed an Advanced Fellowship in Transplant Hepatology at BIDMC, and previously completed a Fellowship in Gastroenterology and Hepatology at Yale University School of Medicine, New Haven, CT. In addition to seeing patients in the Liver Center, Liver Transplant Center and Endoscopy Suite, Dr. Cohen directs the hepatology educational curriculum for BIDMC residents, fellows and faculty. His area of clinical research in the complications of portal hypertension.

Robert Cohen, M.D. is Assistant Professor of Medicine at Harvard Medical School, and an attending nephrologist at Beth Israel Deaconess Medical Center. A graduate of Temple University, he went on to Beth Israel Hospital for fellowship in nephrology, and obtained his masters in clinical epidemiology from the Harvard School of Public Health. His areas of interest are diabetic nephropathy, the pathogenesis of lupus nephritis, and IgA nephropathy.

David H. Evans, PhD. is Professor Emeritus of Zoology at the University of Florida. He served as Professor and Chair of the Department of Biology at the University of Miami, Florida, and subsequently Professor and Chair of the department of Zoology at the University of Florida from 1982 to 1985 and 2001-2006. Dr. Evans has also served as Director of the Mt. Desert Island Biological Laboratory. In addition to many teaching awards, Dr. Evans was named the 2008 Krogh Distinguished Scientist by the Comparative and Evolutionary Physiology Section of the American Physiological Society. He is the Editor of three editions of "The Physiology of Fishes", published by CRC Press and is currently editing chapters for a new book "Osmotic and Ionic Regulation: Cells and Animals" for CRC Press. His current research interests center on the hormonal and paracrine control of fish gill perfusion and epithelial transport, specifically the roles of local signaling agents such as nitric oxide, endothelin, and prostaglandins.

Marianne A. Grant, PhD is a structural biologist at the Beth Israel Deaconess Medical Center and an Instructor in Medicine at Harvard Medical School. She received her PhD from Brown University within the Pharmacology, Biotechnology and Physiology Department studying neuromuscular and neuronal ion channel structure and function. She went on in postdoctoral research to study the structure and function of pro- and anticoagulant proteins in the Division of Hemostasis and Thrombosis at the BIDMC. Her current research focus in the Division of Molecular and Vascular Medicine is the elucidation of molecular mechanisms underlying signal transduction and gene regulation pathways that are of central importance in vascular biology and disease pathology.

Susan J. Hagen, PhD is a gastric physiologist and Director of the Histology, Confocal, and Electron Microscopy core facilities at the Beth Israel Deaconess Medical Center and an Associate Professor of Surgery at Harvard Medical School. She also serves as the Associate Vice-chair for Research in Surgery at BIDMC. Dr. Hagen received her PhD from Michigan State University in Developmental Genetics (Zoology) and then did postdoctoral training in cell biology at Johns Hopkins University School of Medicine. Her current research focus is in mechanisms that regulate initiating preneoplastic changes in the stomach leading to gastric cancer development. She has also been instrumental in elucidating mechanisms by which K+-channels regulate gastric acid secretion and the way in which ammonia affects acid secretion and cell death mechanisms in the stomach.

Carl J. Hauser, MD is a trauma surgeon and intensivist in the division of trauma surgery and critical care at Beth Israel Deaconess Medical Center. He received his MD degree from New York University Medical School and trained in Surgery and Critical Care Medicine at Harbor/UCLA Medical Center. He has been an academic trauma surgeon and intensivist for over 25 years, having served on the faculties of the University of Southern California, University of Mississippi, New Jersey Medical School and finally Harvard Medical School. Dr. Hauser has devoted his research to the study of the effects of shock and tissue injury on metabolic and immune function in critically ill patients. His laboratory's work on the role of mitochondrial molecular signatures in innate immunity has received wide attention as a landmark change in our understanding of how injury and sepsis cause inflammation. His work has also received wide attention as a groundbreaking insight into basic mechanisms of clinical inflammation and is widely accepted as creating a "paradigm shift" that will contribute to our understanding of a wide variety of inflammatory disease processes in the future.

Shoshana J. Herzig, M.D., M.P.H. is an Instructor in Medicine at Harvard Medical School and a clinician-investigator within the Division of General Medicine's Section of Hospital Medicine at Beth Israel Deaconess Medical Center. She completed a Master's in Public Health Degree at Harvard School of Public Health, receiving advanced training in pharmacoepidemiology, and a General Medicine Fellowship through Harvard Medical School. Her research focuses on the interplay between medication decisions and adverse outcomes in the hospital setting, in an effort to reduce nosocomial complications, and promote evidence-based prescribing practices.

Warren G. Hill, PhD. is an Assistant Professor of Medicine at Harvard Medical School and a member of the Division of Nephrology at the BIDMC. Dr. Hill's major research interest is epithelial physiology. His doctoral research project focused on the pathophysiology of cystic fibrosis. Following a postdoctoral fellowship in the laboratory of Ray Frizzell, Chairman of Cell Biology and Physiology at the University of Pittsburgh, Dr. Hill moved into the area of epithelial membrane biophysics with Mark Zeidel; then Chair of Medicine at University of Pittsburgh Medical School. Since then, his research efforts have focused on understanding epithelial barrier membrane function in a range of experimental systems which include the mammalian bladder, the plasma membrane of the *Xenopus* oocyte, cell culture models of the kidney and gill epithelia of marine animals such as sharks, flounder and eels. The eel is a particularly interesting creature since it is euryhaline – it can adapt to life in either sea water or fresh. The overarching goal of these studies in comparative physiology has been to understand mechanisms of osmoregulation within organisms exposed to very different environments.

Bryce MacIver, Ph.D. is an Instructor in Medicine at Harvard Medical School and a member of the Nephrology Division at BIDMC. Dr. MacIver researches transporter protein function in relation to epithelia physiology, specifically urea and water channels. He began his research career in bacterial molecular genetics and his PhD was attained studying a myosin gene in the fruitfly *D. melanogaster*. He undertook postdoctoral studies at Penn State University in projects related to *D. melanogaster*. He then joined Dr. Mark Zeidel's group in Pittsburgh to begin studies on epithelial transporter proteins, which he now continues at BIDMC.

John C. Mathai, Ph.D. is an Assistant Professor of Medicine at Harvard Medical School and a member of Division of Nephrology at the BIDMC. His research is focused on understanding the mechanisms of water and solute transport across membranes and proteins such as aquaporins and urea transporters. Recent interests include development of novel techniques to measure gas transport across artificial membranes and tissues.

Federica del Monte, M.D., Ph.D. is an Assistant Professor of Medicine at Harvard Medical School and a member of the Cardiology Division at the BIDMC and MGH. Her research is focusing on understanding the mechanisms of heart failure and specifically of idiopathic Dilated Cardiomyopathy with particular focus on protein misfolding. Similarly to Alzheimer disease and other chronic diseases (e.g. diabetes, cystic fibrosis), protein misfolding seems also to be at the basis of certain forms of iDCM. Her research interest is also in the genetic bases of the disease.

Patricio Silva, M.D. is Professor of Medicine and Medical Director of the Kidney transplant Program and Director of the Nephrology Training Program at Temple University and Temple University Hospital. He is a member of the Section of Nephrology and Kidney Transplantation at Temple University. He was formerly the Chief of the Division of Nephrology at The New England Deaconess Hospital and The Joslin Diabetes Center and the Section of Nephrology and Kidney Transplantation at Temple University. Dr. Silva has been interested in evolutionary and comparative physiology for close to 40 years with his main interest on the regulation of chloride transport.

Richard Solomon, M.D. is Professor of Medicine and Director of the Division of Nephrology at the University of Vermont and Fletcher Allen Health Care. He was a scientist at the MDIBL for 23 years working with Drs. Frank Epstein and Patricio Silva. His current research interests include acute kidney injury and contrast-induced nephropathy.

Mark L. Zeidel, M.D. is Herrman Ludwig Blumgart Professor of Medicine at Harvard Medical School and Physician-in-Chief and Chairman of the Department of Medicine at the BIDMC. He was formerly chief of the Chair of the Department of Medicine at the University of Pittsburgh. Dr. Zeidel's research interests include epithelial biology and water transport.

Participants

Medical Residents:

Korson, Andrew
Germansky, Katherine
Coronado, Michael
Hassan, Zena
Goldman, Jonathan
Babula, Bracken
Lamour, Isabel
Obuch, Joshua
Reigh, Erin
William, Jeffrey
Ford, Coleen
Kothari, Darshan
Govindan, Sujeet
Pleet, Julia
Reddy, Sheela
Sriwattanakomen, Roy
Sukhul, Nidhi
Horton, Devin

Surgical Residents:

Feng, Christina
Kamine, Tovy
Linsk, Ali
Lee, Eliza

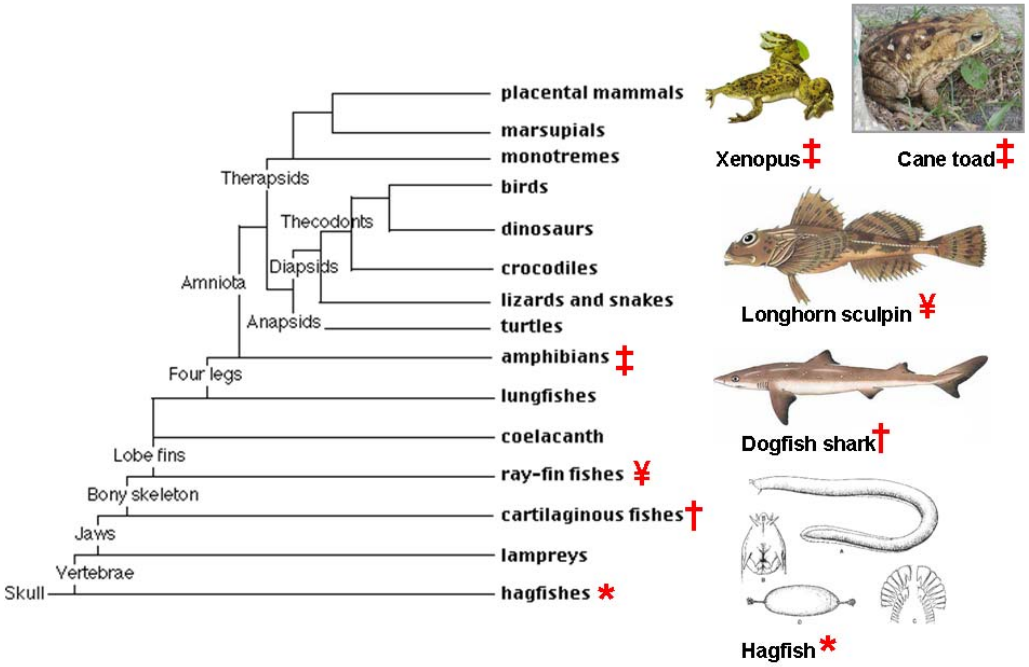
Groups and Schedule

	Module 1 Sun/Mon	Module 2 Tues/Wed	Module 3 Thurs/Fri
Bracken Babula	Cardiac	Hematology	Water Balance
Isabel Lamour	Water Balance	Hematology	Cardiac
Joshua Obuch	Cardiac	Secretory	Vascular Tone
Jeffrey William	Secretory	Gastric Acid	Vascular Tone
Coleen Ford	Water Balance	Hematology	Cardiac
Darshan Kothari	Hematology	Secretory	Cardiac
Sujeet Govindan	Hematology	Gastric Acid	Water Balance
Julia Pleet	Gastric Acid	Water Balance	Secretory
Sheela Reddy	Water Balance	Cardiac	Hematology
Nidhi Sukul	Cardiac	Secretory	Hematology
Roy Sriwattanakomen	Hematology	Cardiac	Secretory
Michael Coronado	Vascular Tone	Water Balance	Hematology
Katie Germansky	Gastric Acid	Water Balance	Cardiac
Jonathan Goldman	Vascular Tone	Cardiac	Secretory
Andrew Korson	Secretory	Vascular Tone	Hematology
Zena Hassan	Water Balance	Vascular Tone	Gastric Acid
Erin Reigh	Vascular Tone	Hematology	Secretory
Eliza Lee	Secretory	Gastric Acid	Vascular Tone
Ali Linsk	Gastric Acid	Cardiac	Water Balance
Tovy Kamine	Secretory	Vascular Tone	Gastric Acid
Christina Feng	Hematology	Water Balance	Gastric Acid
Devin Horton	Cardiac	Secretory	Water Balance

Overview of Week

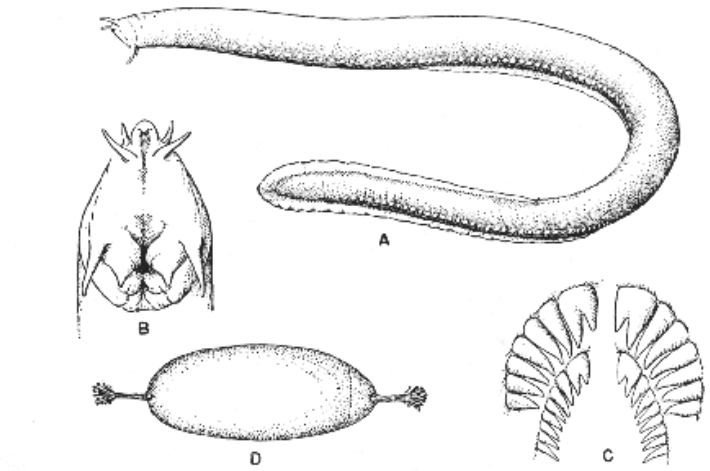
Day	Activity
Arrival: Saturday August 25	
Saturday, August 25– 8:00 PM	Barbeque – Co-op Dining Hall Lawn
Saturday, August 25– 9:00 PM	All groups – Orientation and Opening Talk, Introduction of Lab Rotations, Maren Auditorium, MDIBL
	Module 1
Sunday, Aug 26 – 8:30 AM, PM and evening	Rotations for Module 1
Monday, Aug 27 – 8:30 AM	Rotations for Module 1 (cont'd)
Monday, Aug 27 – 10:00 am	Large lab meeting
Monday, Aug 27 – 12:30 PM	Lunch
Monday, Aug 27 – 2:30 PM	Free time
	Module 2
Tuesday, Aug 28 – 8:30 AM, PM and evening	Rotations for Module 2
Wednesday, Aug 29 – 8:30 AM	Rotations for Module 2 (cont'd)
Wednesday, Aug 29 – 10:00 am	Large lab meeting
Wednesday, Aug 29 – 12:30 PM	Lunch
Wednesday, Aug 29 – 2:30 PM	Free time
	Module 3
Thursday, Aug 30– 8:30 AM, PM and evening	Rotations for Module 3
Friday, Aug 31 – 8:30 AM	Rotations for Module 3 (cont'd)
Friday, Aug 31 – 10:00 am	Large lab meeting
Friday, Aug 31 – 12:30 PM	Lunch
Friday, Aug 31 – 2:30 PM	Free time
Friday, Aug 31Evening	Lobster bake
Departure: Saturday September 1	Checkout by 10am

MEET THE ORGANISMS



Above is a **phylogenetic tree**, which is commonly used to show the evolutionary interrelationships among present day organisms. The major species you will be working with are indicated on the right.

Meet the Organisms



Hagfish (*Myxine glutinosa*)

Despite their name, there is some debate about whether they are strictly fish (as there is for lampreys), since they belong to a much more primitive lineage than any other group that is commonly defined fish (Chondrichthyes and Osteichthyes). Their unusual feeding habits and slime-producing capabilities have led members of the scientific and popular media to dub the hagfish as the most "disgusting" of all sea creatures.[1][2][3]

Hagfish are long, vermiform and can exude copious quantities of a sticky slime or mucus (from which the typical species *Myxine glutinosa* was named). When captured and held by the tail, they escape by secreting the fibrous slime, which turns into a thick and sticky gel when combined with water, and then cleaning off by tying themselves in an overhand knot which works its way from the head to the tail of the animal, scraping off the slime as it goes. Some authorities conjecture that this singular behavior may assist them in extricating themselves from the jaws of predatory fish. However, the "sliming" also seems to act as a distraction to predators, and free-swimming hagfish are seen to "slime" when agitated and will later clear the mucus off by way of the same travelling-knot behavior.

Hagfish have elongated, 'eel-like' bodies, and paddle-like tails. Colors depend on the species, ranging from pink to blue-grey, and may have black or white mottling. Eyes may be vestigial or absent. The hagfish has no true fins or jaws, and has six barbels around its mouth and a single nostril. Instead of vertically articulating jaws like Gnathostomata (vertebrates with jaws), they have a pair of horizontally moving structures with toothlike projections for pulling off food. There are typically short tentacle-like protrusions around the mouth.

Hagfish enter both living and dead fish, feeding on the insides (polychaete marine worms are also prey). While having no ability to enter through skin, they will often enter through current openings such as the mouth, gills or anus. They tend to be quite common in their range, sometimes becoming a nuisance to fishermen by devouring the catch before it can be pulled to the surface. Not unlike leeches, they have a sluggish metabolism and can go months between feedings.

Hagfish average about half a meter (18 inches) in length; *Eptatretus goliath* is the largest known, with a specimen recorded at 127 cm, while *Myxine kuoi* and *Myxine pequenoi* seem to reach no more than 18 cm. An adult hagfish can secrete enough slime to turn a large bucket of water into gel in a matter of minutes.

There has been long discussion in scientific literature about the hagfish being non-vertebrate. Given their classification as Agnatha, Hagfish are seen as an elementary vertebrate in between Prevertebrate and Gnathostome. Thus, their classification is as an extremely primitive Vertebrate.

They are part of the subphylum *Vertebrata* so, taxonomically speaking, they are vertebrates.

They do not have vertebrae so, anatomically, they're not vertebrates. Recent molecular biology analysis tend to classify hagfish as vertebrates (see references), their molecular evolutive distance from *Vertebrata (sensu stricto)* being short. A single fossil of hagfish shows that there has been little evolutionary change in the last 300 million years.

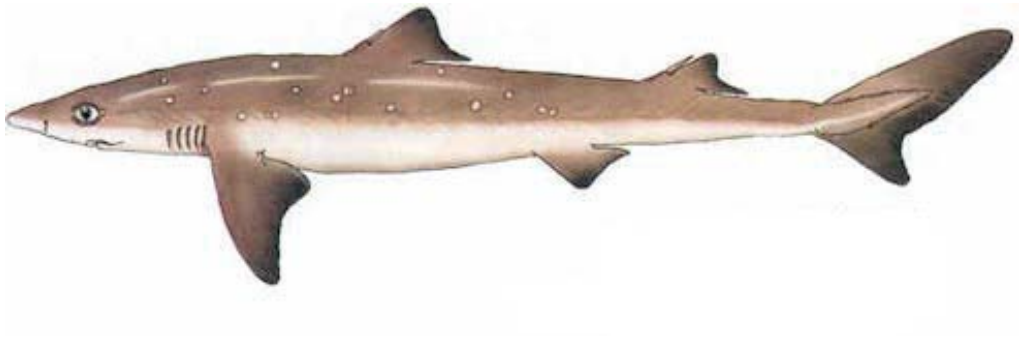
The circulatory system of the hagfish has both closed and open blood vessels, with a heart system that is the most primitive of all vertebrates, bearing some resemblance to that of some worms. This system comprises a "brachial heart", which functions as the main pump, and three types of accessory hearts: the "portal" heart(s) which carry blood from intestines to liver; the "cardinal" heart(s) which move blood from the head to the body, and the "caudal" heart(s) which pump blood from the trunk and kidneys to the body. None of these hearts are innervated, so their function is probably modulated, if at all, by hormones.

Very little is known about Hagfish reproduction. In some species, sex ratio can be as high as 100:1 in favour of females. In other species, individual hagfish which are hermaphroditic, with both ovaries and testes, but the female gonads remain non-functional until the individual has reached a particular stage in the hagfish lifecycle, are not uncommon. Females typically lay 20-30 yolky eggs, that tend to aggregate due to the presence of Velcro-like tufts at either end. Hagfish do not have a larval stage, in contrast to lampreys, which have a long larval phase.

Hagfish are eaten in Japan and South Korea, and their skin is made into "eel leather" (used for so-called "eelskin" products) in Korea.

In recent years hagfish have become of special interest for genetic analysis investigating the relationships among chordates. It has also recently been discovered that the mucus excreted by the hagfish is unique in that it includes strong, threadlike fibres similar to spider silk. What is interesting about hagfish slime is that it is fibre-reinforced. No other slime secretion known is reinforced with fibres in the way Hagfish slime is. The fibres are about as fine as spider silk (averaging two micrometers), but can be twelve centimeters long. When the coiled fibres leave the Hagfishes' 'slime' gland, they unravel quickly to their full length without tangling. Research continues into potential uses for this or a similar synthetic gel or of the included fibres. Some possibilities include new biodegradable polymers, space-filling gels, and as a means of stopping blood flow in accident victims and surgery patients.

From http://en.wikipedia.org/wiki/Hagfish



Dogfish shark (*Squalus acanthias*)

The bodies of dogfish shark are dark gray above and white below, often with white spotting on the sides. They are marketed as "rock shark", "rock cod" or "rock salmon" and are often eaten in Europe and used for English fish and chips. This species is called dogfish because they are gregarious and travel in packs of hundreds to thousands of individuals. From a morphological, experimental, fisheries, and biological perspective, it is the most written about and best known of living sharks.

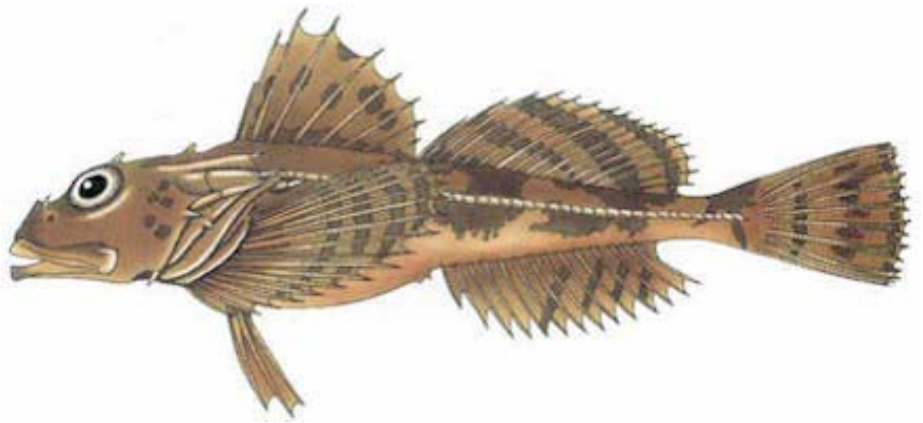
Dogfish often occur in schools segregated by size and sex, although they are also found as solitary individuals. Age and length at maturity appears to vary greatly with region. In the northeast Pacific Ocean estimates of the age at maturity for female spiny dogfish have ranged from 20 years and 3 feet (92cm) to 35.5 years and 3.1 feet (94cm) (Saunders and McFarlane 1993, Bonham et al. 1949). Dogfish are believed to live 25-100 years. The females are larger than the males, and produce from 2-11 (average 7) pups that are between 8-12 inches (20-30 cm) long. They can grow to around 5 feet (1.5m) and weigh about 20 pounds (9kg). They are ovoviviparous (produce eggs that hatch within the females body) and bear live young. The gestation period is the longest of any vertebrate, about 22-24 months after a winter mating (Saunders and McFarlane 1993).

The spiny dogfish is a voracious predator that feeds primarily on bony fishes. They are known to prey heavily on schools of spawning capelin, and congregations of dogfish are often associated with herring returning to coastal waters of British Columbia. During a capelin spawning event on the Copper River flats (Alaska) during the 1998 salmon gillnet fishery, dogfish were so abundant that they often plugged gillnets and disrupted the fishery. Their principal food appears to be herring, sandlance, smelt, and euphausiids. Their diet also includes some 27 other fish species and 13 varieties of invertebrates, many of which are commercially important (Hart 1980). Dogfish are also known to prey on juvenile salmon.

The spiny dogfish is believed to be the worlds' most abundant shark and is the predominant shark species in Alaska. Dogfish occur in depths from the surface down to 3000 feet (900m). They are adaptable predators and are often found in brackish waters around the mouths of estuaries and feeding over tidal flats. They occur worldwide in temperate and sub-arctic waters, and in the temperate and sub-arctic latitudes of the North Atlantic and North Pacific oceans. They favor a temperature range with a minimum of 450F (70C) and a maximum of 590F (150C) and make latitudinal and depth migrations to stay within their optimum range. In the eastern Pacific Ocean they are found off Chile and from central Baja California to Alaska and to Japan. Some tagged sharks in the eastern North Pacific have traveled long distances, in one case a dogfish swam

4039 miles (6500km) to Honshu, Japan where the shark was captured 7 years after it was tagged off the coast of Washington state. One specimen was caught in a salmon gillnet during August of 1984 near Kotzebue, Alaska, far north of it's usual range. They may occur year round in Alaskan's southern coastal waters but are most common from spring to fall.

From http://www.conservationinstitute.org/ocean_change/predation/spinydogfish.htm



Longhorn sculpin (*Myoxocephalus octodecemspinosus*)

The longhorn, like other sculpins, varies in color with its surroundings. It grows to a maximum length of about 18 inches, but only a few of them are more than 10 to 14 inches long. A 10-inch fish weighs about ½ pound, one 12 inches long about 1 pound. This sculpin is a nuisance to cunner and flounder fishermen. It often is bothersome to the angler to unhook when it spreads its needle-sharp spines and erects its spiny dorsal fin. It grunts when pulled out of the water and bites on any bait. It is caught anywhere and everywhere along the entire coast line of the Gulf of Maine. The only commercial value this sculpin has had in our Gulf was as bait for lobster pots, for which they were speared formerly in some localities, and caught on hook and line in others. But very few of them are now used in this way.

From: http://www.gma.org/fogm/M_octodecimspinosus.htm

Cane Toad (*Bufo marinus*)

Bufo marinus is also known as the cane toad or marine toad. Its natural range is from the Rio Grande Valley of Texas south to the Central Amazon and southeastern Peru. It is a tropical species that prefers forested areas with semi-permanent water nearby. *B. marinus* has a grey olive brown dorsal skin with many warts ending in dark brown caps. The ventral skin tends to be a whitish yellow with dark brown speckles or mottles and is granular. *B. marinus* possesses huge paratoid glands stretching from the anterior side of the tympanum to halfway down the back. A high bony ridge meets at the snout between the nostrils. *B. marinus*, like other nocturnal species, has horizontal pupils. *B. marinus* can reach a maximum length of 238 millimeters, although generally is approximately 150 to 175 millimeters. The Cane Toad is a prolific breeder. Females are able to reproduce after their second year. Eggs are laid in long jelly-like strings on rocks, debris, or emergent vegetation in excess of 30,000 at a time. Males are able to reproduce as both sexes because they possess a rudimentary ovary that becomes operative if their testes are removed or damaged. The cane toad sits in an upright position when it moves, it hops in short fast hops. During cold or dry seasons it will remain inactive in shallow excavations beneath ground cover. When confronted by a predator, it is able to "shoot" bufotoxin from the paratoid and other glands on the back in the form of white viscous venom. This venom is largely comprised of cardioactive substances. These bufotoxins can lead to profuse salvation, twitching; vomiting; shallow breathing and collapse of the hind limbs if bitten, ingested, or when in contact with mucous membranes. This toxin can cause temporary paralysis or even death in some small mammals and predators. *B. marinus* has a voracious appetite. It forages primarily nocturnally in mature forests and roadways. It feeds on ants, beetles, and earwigs in southern Florida, but has been found with dragonflies, grasshoppers, truebugs, crustaceans, gastropods, plant matter and even dog and cat food in their stomachs. This toad is considered the most introduced amphibian in the world as a method of agricultural pest control.

[Prinsen", a specimen kept as a pet in Sweden, is listed by the *Guinness Book of Records* as the largest recorded specimen, which weighed 2.65 kilograms (5.84 lb) and measured 38 centimetres (15 in) from snout to vent, or 54 centimetres (21 in) when fully extended]

From: http://en.wikipedia.org/wiki/Cane_Toad;
http://animaldiversity.ummz.umich.edu/site/accounts/information/Bufo_marinus.html

African clawed frog (*Xenopus laevis*)

Xenopus laevis is a species of South African aquatic frog of the genus *Xenopus*. It is up to 12 cm long with a flattened head and body but no tongue. Its name derives from its three short claws on each of its hind feet, which it probably uses to stir up mud to hide it from predators. *Xenopus* are a popular model system for gene and protein expression and knockdown studies. At 1 mm diameter, *Xenopus* oocytes are very large cells which are easy for scientists to culture and use in experiments. RNA from other organisms can be injected into the large oocytes and the resulting expression studied via molecular biology techniques or through electrophysiology experimentation. Gene expression can be knocked down or splicing modified using Morpholino antisense oligos injected into *Xenopus* oocytes (for whole-body effects) or early embryos (for effects in the daughter cells descended from the injected cell). Cytoplasmic extracts made by centrifuging *Xenopus* eggs or embryos (allowing direct addition or depletion of proteins) can recapitulate a wide range of complex cellular processes including nuclear envelope formation, DNA replication and spindle assembly.

From: http://en.wikipedia.org/wiki/Xenopus

Classifying Fishes

Three classes:

1. **AGNATHA.** Fish of the class agnatha ("no jaw") are the most "primitive" of the fishes; they lack a jaw and a bony skeleton. The hagfish and the lamprey are the only living representatives of this once large class. As they lack true bones, these fish are very flexible, the hagfish can actually tie itself in a knot to rid itself of a noxious slime it can produce to deter predators. They have a smooth, scaleless skin and are soft to the touch. In place of the jaws is an oral sucker in the center of which is the mouth cavity. Many of the agnathas are highly predatory, attaching to other fish by their suckerlike mouths, and rasping through the skin into the viscera of their hosts. The juvenile lamprey feeds by sucking up mud containing micro-organisms and organic debris - as did the primitive agnatha. Agnathas are found in both fresh and salt water and some are anadromous [living in both fresh and salt water at different times in its life cycle]. The hagfish has no eyes, while the lamprey has well-developed eyes.

2. **CHONDRICHTHYES.** Members of the class Chondrichthyes ("cartilage-fish") include the sharks, skates, rays, and ratfish. These fish have a cartilaginous skeleton, but their ancestors were bony animals. These were the first fish to exhibit paired fins. Chondrichthyes lack swim bladders, have spiral valve intestines, exhibit internal fertilization, and posses 5-7 gill arches (most have 5). They have cartilaginous upper and loosely attached lower jaws with a significant array of teeth. Their skin is covered with teethlike denticles which gives it the texture and abrasive quality of sandpaper.

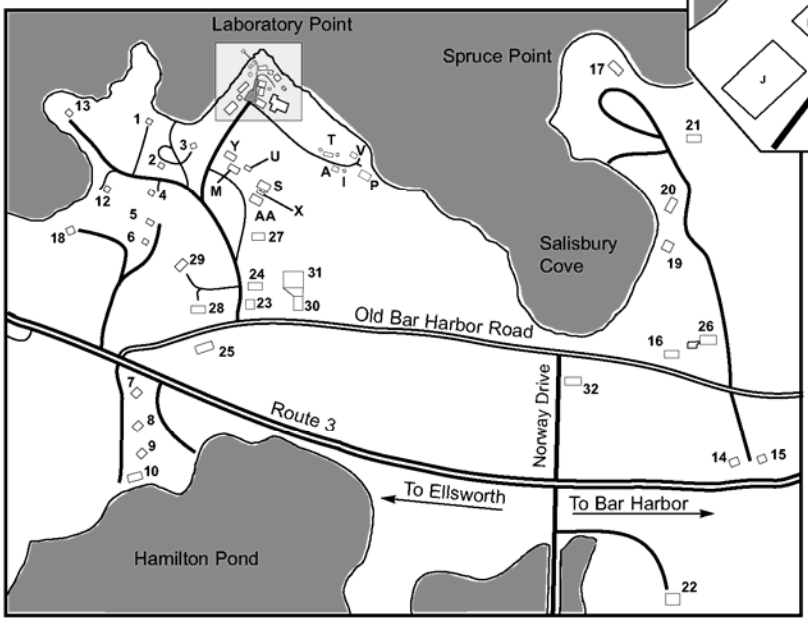
3. **OSTEICHTHYES**. The bony fish comprise the largest section of the vertebrates, with over 20,000 species worldwide. They are called bony fish because their skeletons are calcified, making them much harder than the cartilage bones of the chondrichthyes. The bony fishes have great maneuverability and speed, highly specialized mouths equipped with protrusible jaws, and a swim bladder to control buoyancy. The bony fish have evolved to be of almost every imaginable shape and size, and exploit most marine and freshwater habitats on earth. Many of them have complex, recently evolved physiologies, organs, and behaviors for dealing with their environment in a sophisticated manner.

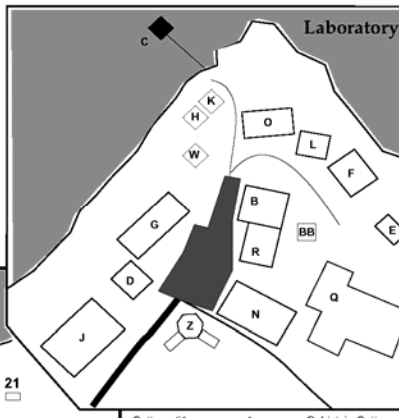
Source: http://www.geocities.com/aquarium_fish/fishclasses.htm

The Mount Desert Island Biological Laboratory

Salisbury Cove, Maine

rev. May 2007





Cottage #1	1	Goldstein Cottage
Cottage #2	2	White Cottage
Cottage #3	3	Rieck Cottage
Cottage #4	4	Silva Cottage
Cottage #5	5	Wilde Cottage
Cottage #6	6	Eden I Apts. A & B
Cottage #7	7	Eden II Apts. A & B
Cottage #8	8	Emery Apts./Dorm
Cottage #9	9	Bow End
Cottage #10	10	Burger Dorm
Bevelander Cottage	12	Bowen Hall Dorm
Forster Cottage	13	Dining Hall (Co-op)
Bymes Cottage	14	Dahlgren Hall
Hoskins Cottage	15	Maren Auditorium
Doyle Cottage	16	US Post Office
Epstein Cottage	17	

Laboratory Area

Chemical Shed	A	Neal
Conference Room	B	Oaks Dorm
Dock	C	Office
Fish Shed	D	New Lab Building
Forster	E	Smith
Halsey	F	Spruce Dorm
Hegner	G	Studies
Instrument Shed	H	Storage Shed
Isotope Shed	I	Treatment Plant
Kamofsky	J	Rappaport Lab
Kidney Shed	K	Volleyball Court
Lewis	L	Tank House
Library	B	Myers Aquarium
Maintenance	M	Birch Hall
Marshall	N	Gull Shed

Maps and Driving Directions

From Boston, Bangor and points in between:

1. *From Boston*: I-95 North from Boston to Bangor area (approx 215 miles).

2. Exit to I-395 (exit 182A).

3. Exit to Route 1A (exit 6A--Coastal Route/Ellsworth/Bar Harbor)

4. Take Route 1A from Bangor to Ellsworth (approx 24 miles)

5. Route 1 and 3 merge near the McDonald's

6. Continue on Route 3 to Mount Desert Island. At the of the head of the island (Exxon gas station), it's approx. four miles to Old Bar Harbor Road. Old Bar Harbor Road will intersect with Rt. 3 on your left.

7. Turn left onto Old Bar Harbor Road and follow approximately 1/8 mile to Dahlgren Hall/Maren

Conference Center (yellow & white building with auditorium attached located on the left just beyond the entrance to the Lab)

Parking: is located down the laboratory driveway toward the office. Please note that certain areas are restricted. Overflow parking is located curbside on Old Bar Harbor Road (on the conference center side only, please), in the small gravel parking lot adjacent to the building, or in the field across the street.

Housing

Birch 2
Nidhi Sukul
Erin Reigh

Birch 3
Zena Hassan
Sheela Reddy

Birch 5
Julia Pleet
Katie Germansky

Birch 6
Ali Linsk
Eliza Lee

Birch 7
Christina Feng

Birch 8
Colleen Ford
Isabel Lamour

Spruce 1
Darshan Kothari
Sujeet Govindan

Spruce 2
Jeffrey William
Tovy Kamine

Spruce 3
Roy Sriwattanakomen
Jonathan Goldman

Spruce 4
Bracken Babula
Andrew Korson

Spruce 5
Michael Coronado
Josh Obuch

Spruce 6
Devin Horton

BIDMC Course on Comparative Physiology

Gastric Acid Module

Gastric Acid Secretion

Goals:

1. Understand the mechanisms of gastric acid secretion in frog and human gastric mucosa
2. Understand the structure and function of H-K ATPase in the parietal cell
3. Understand the effects of substances and processes that inhibit and stimulate gastric acid secretion
4. Understand the properties of H2 blockers and PPIs that make them effective in management of medical and surgical diseases that involve acid hypersecretion, such as GERD and PUD.

Background

Peptic ulcer disease and gastroesophageal reflux disease (GERD) are common and important causes of morbidity and mortality and are both associated with gastric acid hypersecretion. Additionally, acid-induced stress ulceration and upper GI bleeding are common following trauma and in patients with sepsis and septic shock, leading to increased mortality in this population. Suppression of gastric acid secretion is an integral part of management of both PUD and GERD as well as an important aspect of the International Guidelines for the Management of Severe Sepsis and Septic Shock. Drugs that are used to treat acid hypersecretion (PPIs and H2 blockers) are among the best selling drugs worldwide. In order to understand the mechanisms of action of these drugs, one must first understand the mechanisms of gastric acid secretion.

In order to enhance our understanding of the mechanisms of gastric acid secretion and the drugs which inhibit acid secretion, we will perform several experiments on frog gastric mucosa. Frog gastric mucosa has a baseline acid secretion rate that can be measured with the use of an Ussing chamber (see full description and diagram below and in attached references). Once baseline data is obtained, substances that stimulate gastric acid secretion (such as histamine, carbachol, gastrin) and substances that inhibit gastric acid secretion (such as PPIs and H2 blockers) can be added with subsequent measurements of gastric acid secretion to determine the effect of these substances. Further characteristics of drug effect such as time to onset, percent change in acid secretion rate, changes in ion transport properties, duration of effect, can also be determined from these experiments.

Learning about the history of the surgical and medical management of GERD and PUD helps us to appreciate the journey of scientific discovery in this field. The first drug shown to reduce gastric acid secretion was deadly nightshade, or belladonna, which contains atropine, a non selective muscarinic antagonist. Unfortunately, side effects including dry mouth, urinary retention, and pupillary dilation with blurred vision made it difficult to tolerate so attention was turned to surgical management. At the end of the nineteenth century, total and partial gastrectomy was introduced by Theodor Billroth. This was later replaced by complete vagotomy and partial vagotomy. Vagotomy enabled the ablation of muscarinic stimulation of acid secretion without the side effects of atropine.

As the mechanism of gastric acid secretion was elucidated by studying chambered frog mucosa, isolated gastric glands, and isolated parietal cells, the major stimuli of gastric acid secretion were identified as

acetylcholine, histamine and gastrin. Subsequent research then focused on developing antagonists to receptors for these hormones to enable a targeted non-surgical approach to management. See below illustration of parietal cell from NEJM article on H2 blockers in 1990.

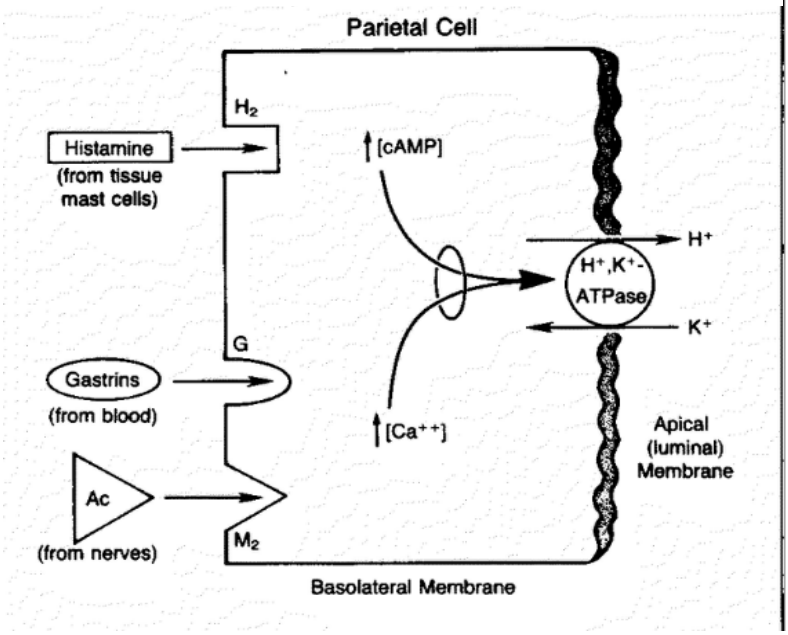


Figure 2. Simplified Model of a Parietal Cell, Containing on Its Basolateral Membrane Separate Receptors for Histamine (H_2), Gastrins (G), and Acetylcholine (Muscarinic$_2$ Receptors [M_2]).

Histamine increases intracellular cyclic AMP (cAMP) concentrations, whereas gastrins and acetylcholine (achéal) increase intracellular calcium. The proton pump (H^+,K^+-ATPase) is located on the apical membrane.

Mild elevations in gastrin were found in PUD and H. pylori infection and the disease of Zollinger Ellison syndrome characterized by excess production of gastrin by neuroendocrine cells was also discovered. Unfortunately, gastrin antagonists were not found to be effective in inhibition of gastric acid secretion. Unexpectedly, H2 blockers were discovered to decrease gastrin but not cholinergic stimulation of acid secretion indicating that the action of gastrin was mediated by release of histamine and muscarinic stimulation was via a different route (later identified as enterochromaffin like cell of gastric mucosa).

In 1976, the electro neutral ATP dependent hydrogen potassium exchanger pump was identified as the mediator of gastric acid secretion. If the pump could be inhibited effectively, hormonal stimulation of the parietal cell would not affect gastric acid secretion. The discovery of the proton pump inhibitor which inhibits the H-K ATPase via covalently bonds thus represented a revolution in management of GERD and PUD. Since PPIs form bonds with the H-K ATPase, their half life of inhibition of gastric acid secretion is much longer than their half life in blood. They are also activated by an acidic pH and tend to accumulate in an acidic environment which specifically targets these drugs to stomach, decreases their adverse effect profile, and expands their therapeutic index. These properties confer a large therapeutic index but also cause a delay in suppression of the pump's activity (3 days to reach steady state). Since they require stimulation of gastric acid secretion, they are more also effective after a meal.

In this module, we will study the mechanisms of gastric acid secretion and the pivotal role of the H-K ATPase pump as well as learn about the mechanisms of action of various stimulants and inhibitors of gastric acid secretion.

Schedule and Outline

Day 1: Stimulation of acid secretion via H2 receptor, M3 receptor, and gastrin receptor signaling.
Morning: Measure baseline gastric acid secretion and the effects of stimulants of gastric acid secretion including histamine, carbachol, histamine + carbachol, and pentagastrin.
Afternoon: Measure baseline gastric acid secretion and then stimulate with histamine +carbachol or forskolin, which increases cAMP and stimulates secretion without H2 receptor ligation. After maximal stimulation is accomplished, add IBMX (a phosphodiesterase inhibitor), which should increase acid secretion further by increasing cAMP. To verify the important role of cAMP in acid secretion, SQ 22536 (a potent and specific cAMP inhibitor) will be added. This drug should block acid secretion by reducing receptor signaling via cAMP.

Day 2: Organize data and presentations

Day 3: Inhibition of acid secretion.
Morning: Measure baseline gastric acid secretion and then stimulate with histamine + carbachol. Once maximal secretion is obtained, add omeprazole or cimetidine. Once acid secretion is inhibited fully, wash-out the omeprazole or cimetidine and re-stimulate the tissues with histamine + carbachol.

On companion tissues, do not stimulate but add omeprazole or cimetidine to tissues that have basal secretion only. Measure the rate of acid suppression.

Afternoon: Measure effects of omeprazole, cimetidine, and PD 134,308 (a potent antagonist of pentagastrin) on pentagastrin stimulated gastric acid secretion.

Day 4: Organize data and presentations

Day 5: Ions important for the regulation of acid secretion; Sodium, Potassium, and Bicarbonate.
Morning: Measure baseline gastric acid secretion and then stimulate with histamine + carbachol. Once secretion is maximal, add DIDS. DIDS blocks the sodium/bicarbonate co-transporter. With the other tissue we will use bicarbonate-free solutions to inhibit bicarbonate transport. We will stimulate with histamine + carbachol and measure acid secretion in the absence of bicarbonate.
Afternoon: Measure baseline acid secretion and then stimulate with histamine + carbachol. To one tissue we will add extra potassium (3x; which will increase the acid secretion rate) and to the other tissue we will add chromanol 293B, which is a potent KCNQ1 K channel blocker). Acid secretion will be measured.

Day 6: Organize data and presentations

Schedule overview

Module 1:

AM	1A: Histamine	1B: Carbachol	2A: Histamine + carbachol	2B: Pentagastrin
PM	1A: Histamine + carbachol + SQ 22536/DMSO	1B: Histamine + carbachol + IBMX	2A: Forskolin	2B: Forskolin + IBMX

Module 2:

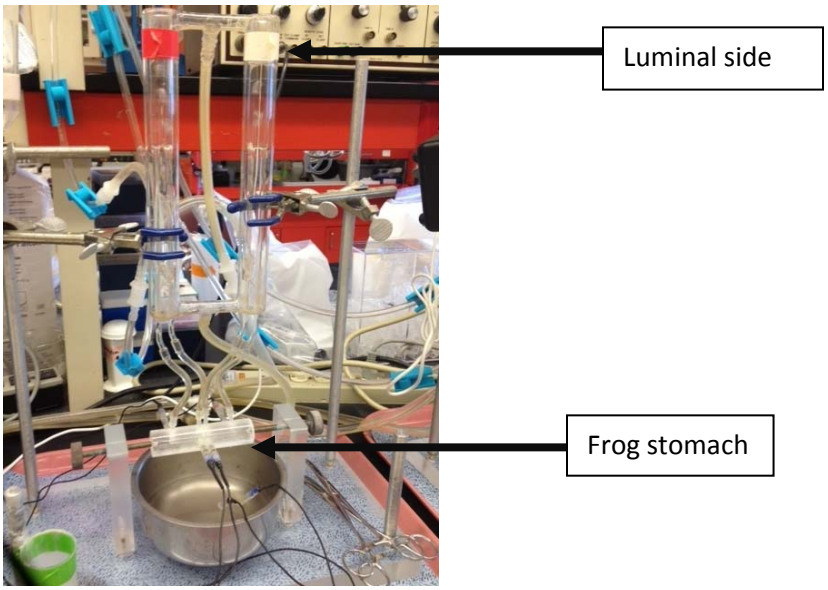
AM	1A: Resting + omeprazole	1B: Histamine + carbachol + omeprazole; Washout omeprazole and re-stimulate with H/C	2A: Resting + cimetidine	2B: Histamine + carbachol + cimetidine; Washout cimetidine and re-stimulate with H/C
PM	1A: Pentagastrin	1B: Pentagastrin plus omeprazole	2A: Pentagastrin	#2B:Pentagastrin + PD 134308

Module 3:

AM	1A: Histamine + carbachol	1B: Histamine + carbachol + DIDS	2A: Histamine + carbachol in regular buffer	2B: Histamine + carbachol in bicarbonate-free buffer
PM	1A: Histamine + carbachol	1B: Histamine + carbachol, chromanol 293B	2A: Histamine + carbachol	2B: Histamine + carbachol, 3-fold higher K+

Experiment protocol for all modules

1. Mount each half of a frog stomach (ie. 1A and 1B) in an Ussing chamber (See picture for experimental setup). White tape marks the luminal side of the setup where acid is secreted from the frog stomach.
2. The pH electrode on the luminal side of the chamber measures the pH. NaOH is used to buffer the amount of acid secreted by the frog stomach. Using this information, we can calculate the acid secretion rate. There are voltage and current electrodes mounted in the front and back of the chamber, which allow us to calculate the resistance (R) using Ohms law (R = V(voltage)/I(current).
3. Measure the change in voltage (which is the "potential difference"(PD) or delta PD) at baseline and at 30 minutes in the presence of a 50 microamp current that is "shot" across the mucosa. Add experimental solution (stimulant, inhibitor, etc) then measure the delta PD and acid secretion rate at 30, 60, 90 and 120 minutes. Using the delta PD measurement (obtained from the chart recorder), the 50 microamp current, and the acid secretion rate, calculate the electrical and secretory properties of the stomach in each experimental condition.



As you are doing the above experiments, consider the following questions.

1. How can I use changes in current and voltage to calculate change in resistance?
2. Why are gastrin inhibitors not effective clinically?
3. What is the effect of omeprazole and cimetidine on gastric acid secretion when first treated with histamine+carbachol vs pentagastrin and why are these different?
4. What are the ion transport mechanisms activated when H+ and Cl- are secreted and why are they necessary?
4. How does potassium channel blockade affect gastric acid secretion?
5. Why does cAMP inhibition effect gastric acid secretion?

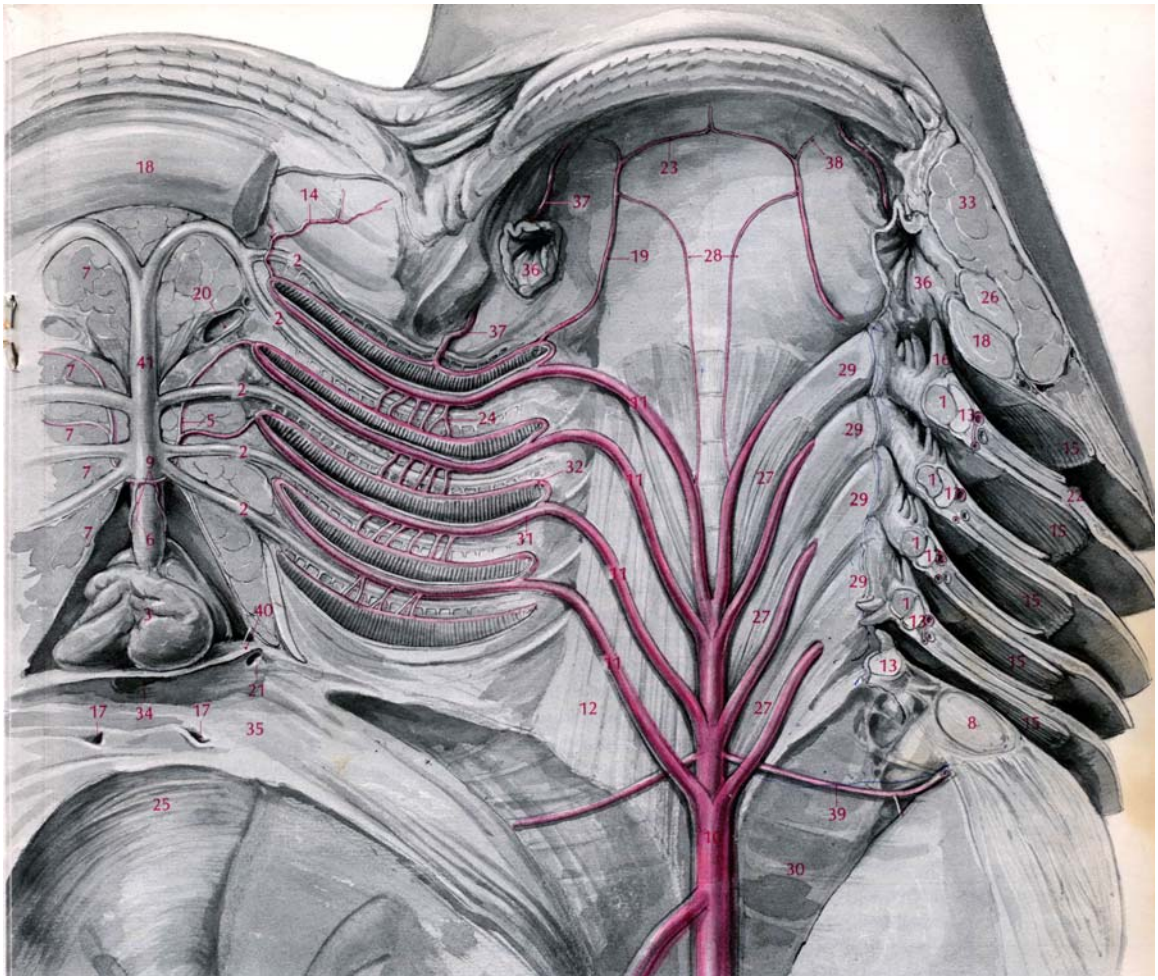
References

1. Shin et al. The gastric HK-ATPase: structure, function and inhibition. Pflugers Arch. January 2009. p. 609-622.
2. Voute, C and Ussing H. Some morphological aspects of active sodium transport. The epithelium of the frog skin. The Journal of Cell Biology Volume 36. 1968, p. 625-638.
3. Shin et al. Molecular mechanisms in therapy of acid-related diseases. Cell Molecular Life Sciences. January 2008. p. 264-281.
4. Oates et al. Histamine 2 receptor antagonists- Standard therapy for acid-peptic disease. NEJM 1990.

BIDMC Course on Comparative Physiology

PHARMACOLOGY OF VASCULAR SMOOTH MUSCLE MODULE

David Evans, Ph.D.
Kelly A. Hyndman, Ph.D.

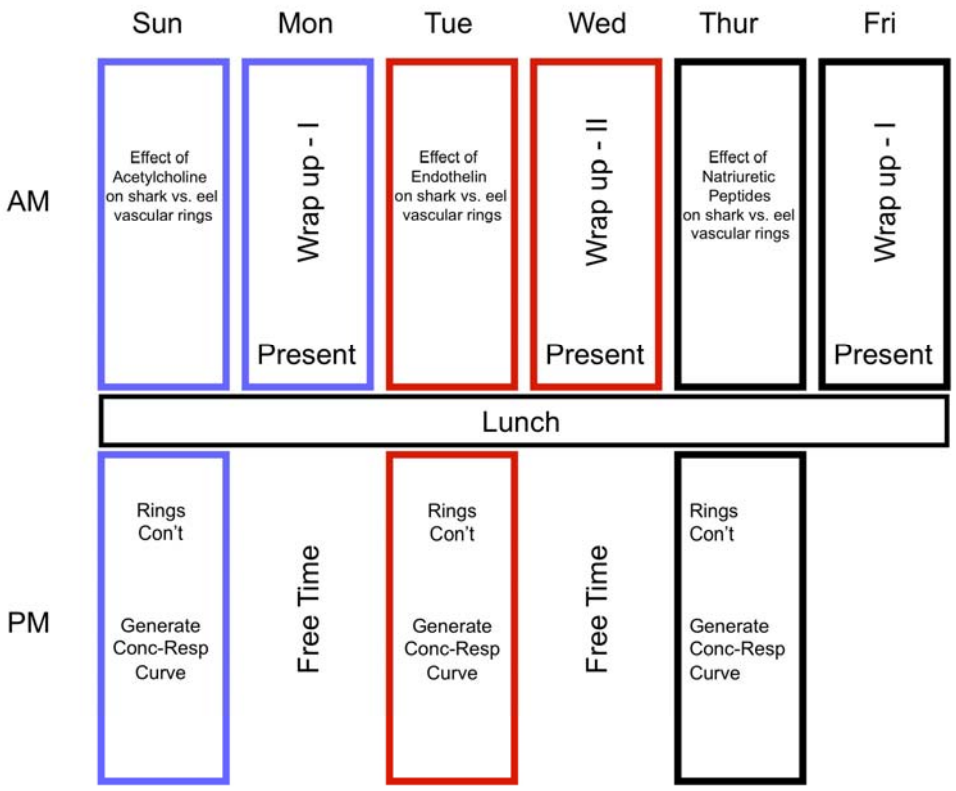


Gilbert, S.G. Pictorial Anatomy of the Dogfish. U. Wash. Press, 1992

GOALS AND SCHEDULE

The Pharmacology of Vascular Smooth Muscle Module of this course is designed to review basic physiologic concepts, use comparative experimental systems to better understand these concepts, and to suggest comparisons with mammalian/human physiology. This module contains three component sections, each complementary but distinct, with the following aims:

1) Investigate the role of the vascular endothelium in the response of isolated VSM from the dogfish shark and American eel to acetylcholine. Is there an endothelium-derived relaxing factor in fishes that is stimulated by ACh?
2) Investigate the vasoactive effects of endothelin on isolated VSM from the dogfish shark and American eel. Are the putative receptors for ET the same in both species?
3) Investigate the vasoactive effects of natriuretic peptides on isolated VSM from the dogfish shark and American eel. Are the putative receptors for NPs the same in both species?



BACKGROUND

Respiratory Gas Exchange in Fishes

Similar to humans, and as discussed in the Introduction to the Hematology module, fishes are also reliant on diffusion to extract necessary oxygen from the surrounding medium. Unlike humans, fish have a more difficult task due to the relatively low oxygen tension in water (ca. 7 ml/l water—compare with oxygen tensions in air). This requires that they move large quantities of water (compare the density of water vs. air) past the gill to provide necessary oxygen to the blood (tuna have about the same BMR as humans), and so fishes expend more energy for respiration than air breathers (ca. 20% of BMR—compare to humans).

Like the mammalian lung, the fish gill epithelium is elaborated into a huge surface area, with numerous filaments on each gill arch, subdivided into small divisions termed lamellae (Fig. 1). (Compare this structure with the human lung) For a series of papers on fish gill form and function, go to:
http://www3.interscience.wiley.com/cgi-bin/jissue/95016487

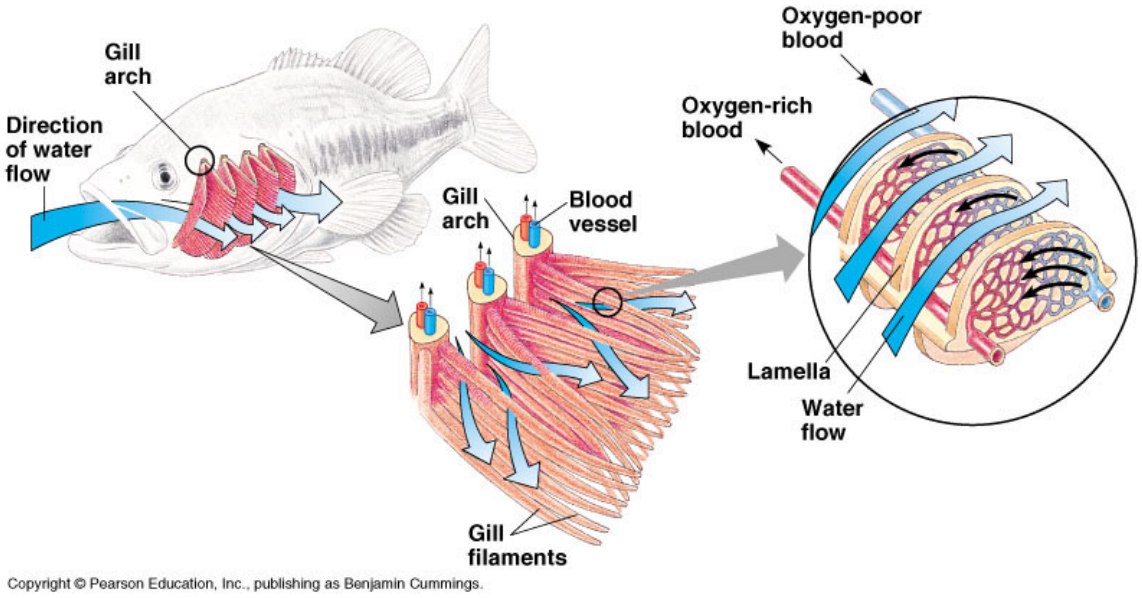


Fig. 1

Note in the right panel of Figure 1 that the perfusion of blood and irrigation of water are in opposite directions, resulting in countercurrent flow—one of the classic cases of this important physiological mechanism. Can you think of any examples of counter-current exchange in the human? This maximizes the extraction of oxygen from the external medium (ca. 75% vs. what in humans?), and it also maximizes excretion of CO_2. What might this mean for fish plasma pH

and bicarbonate levels and for acid-base regulation in fishes vs. acid-base regulation in mammals? Note the blood flow through the outer marginal channels. What might this mean for gas exchange? How might a fish change the surface area for gas exchange? (Think gross morphological changes (move gill arches) vs. microperfusion changes). Does this happen in the human lung?

The Multifunctional Fish Gill

Gas exchange is only one of the four basic physiological processes that are mediated by the fish gill. Because of the very large surface area of the gill epithelium, this organ is the site of passive movements of salts and water down the gradients present because the plasma ionic concentration and content is between that found in seawater and fresh water. Contrary to the situation in mammals, the kidney does not play a major role in osmoregulation in fishes (especially in SW), but specialized cells in the gill epithelium can actively secrete salts in SW and actively extract salts from FW. Some of these ionic movements are associated with excretion of acid vs. base, so (again, contrary to what one sees in mammals) the gill is also the major site for regulation of blood pH. And the major nitrogenous waste product of fishes (like most aquatic animals) is ammonia (why do mammals excreted urea?), which diffuses (as NH_3) or is transported (as NH_4^+) across the gill epithelium. So the gill is not just a gas exchanger!

The intent of this module is to study the putative role of various vascular signaling agents in controlling perfusion of the fish gill vasculature, which could control all of these very important physiological processes. Because gill blood vessels are too small to study individually, we will use vascular rings of ventral aortae as models, with the proviso that various vascular beds might respond to these putative signaling agents in different ways (compare pulmonary and systemic vessel responses on humans), because not all blood vessels express the same receptors for specific vasoactive substances. What effect might this heterogeneity have on microcirculation in fishes, or humans?

Endothelial Control of Vascular Smooth Muscle

Consider the following signaling pathways between mammalian endothelial cells and the underlying vascular smooth muscle.

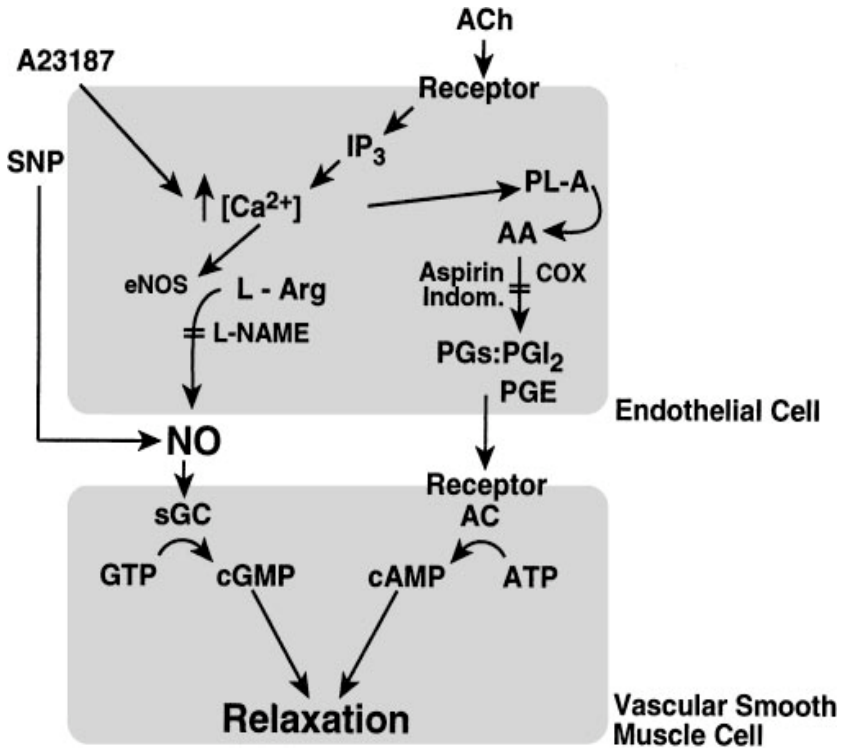
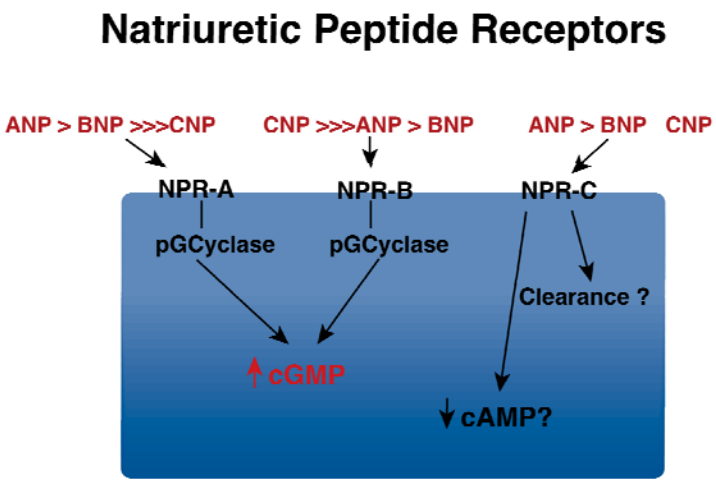


Fig. 1. Nitric oxide (NO) and prostaglandin (PG) signaling pathways involved in endothelium-dependent responses of vascular smooth muscle cells in mammals. ACh, acetylcholine; SNP, sodium nitroprusside; eNOS, endothelial NO synthase; L-Arg, L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; Indom, indomethacin; sGC, soluble guanylyl cyclase; IP_3, inositol trisphosphate; PL-A, phospholipase A; AA, arachidonic acid; COX, cyclooxygenase; PGI_2, prostacyclin; AC, adenylyl cyclase.

Nitric oxide (a gas) and prostaglandins (lipids) are called endothelium-derived relaxing factors (EDRF). Receptors for ACh are found on both the endothelial cells and vascular smooth muscle, so the ultimate effect of ACh stimulation of vascular smooth muscle (dilation vs. constriction) depends on whether the endothelial cells are intact (see Furchgott and Zawadzki, 1980). Our earlier work demonstrated that the shark ventral aorta endothelium apparently does not express ACh receptors and suggests that PGE, not NO, is the EDRF in shark ventral aorta (Evans and Gunderson, 1998). In fact, NO actually constricts the shark VSM from a variety of shark abdominal vessels, including the posterior intestinal vein (Evans, 2001), and eNOS is not in any fish genome database. See the paper by Feletou for a review of NO effects on vascular smooth muscle.

Endothelins are a family of small, paracrine peptides that were first described in cultures of endothelial cells (Yanagisawa et al., 1988). They are produced in a variety of tissues and play roles in regulation of vascular tone, alteration of ion transport, and migration of neural crest cells during craniofacial development. Previous studies have demonstrated both vascular and transport effects in fishes (e.g., Olson et al., 1991; Evans and Harrie, 2001; Evans et al., 2004; Hyndman and Evans, 2007). See papers by Webb and Granger for recent reviews on endothelin.

Natriuretic peptides are a family of small peptides that were first described in rat atria, but have now been found in most tissues, especially the heart, brain, and vascular endothelium. They are diuretic, natriuretic, and vasodilatory. Many studies have demonstrated vasodilation by NPs in fish vasculature (e.g., Olson and Meisheri, 1989; Evans et al., 1993; Evans and Harrie, 2001). See the paper by Rubattu for a recent review of NPs in human physiology.



This week, we will examine the effects of these putative vasoactive agents on isolated vascular rings from the ventral aortae of the dogfish shark (an elasmobranch) and American Eel (a teleost). By the end of the week, you should know the following:

1. Does the endothelium of VSM from the shark and eel contain receptors for acetylcholine?

2. Is nitric oxide vasodilatory in fishes?

3. What is the relative efficacy of ACh in vessels from the shark vs. the eel?

4. What is the effect of endothelin on fish vascular smooth muscle? Does this effect differ in the shark vs. the eel?

5. What is the relative efficacy in the two species?

6. What ET receptors might be involved?

7. What is the effect of natriuretic peptides on the fish vascular smooth muscle? Does this effect differ in the shark vs. the eel?

8. What is the relative efficacy in the two species?

9. What NP receptors might be involved?

GROUP INVESTIGATIONS

Group 1 will generate a concentration-response curve for the vasoactive effect of acethylcholine on shark and eel aortic vascular smooth muscle. By using both intact and endothelium-free tissue, they will test for the presence of ACh receptors on the endothelium vs. the VSM itself. They also will see if NO can stimulate the aortic rings. They will determine if these effects are the same in both species, one an elasmobranch and one a teleost. Their experiments should allow them to determine if there is the classic endothelium-derived relaxation factor in fish vessels.

Group 2 will characterize the response to endothelin and attempt to characterize the specific receptor expressed on endothelium-free VSM of the two fish species, by comparing the effect of ET-1 (acts via both EDNRA and EDNRB) with the effect of SarafotoxinS6c (SRXS6c; specific for EDNRB). They will determine if elasmobranch VSM and teleost VSM express the same type of ET receptor, and the relative affinities of the specific ligands for the receptors.

Group 3 will characterize the response to natriuretic peptides and attempt to characterize the specific receptor expressed on endothelium-free VSM of the two fish species by comparing the effects of atrial natriuretic peptide (NPRA specific) with C-type natriuretic peptide (NPRB specific). They will determine if elasmobranch VSM and teleost VSM express the same type of NP receptor, and the relative affinities of the specific ligands for the receptors.

PROTOCOLS

1. Vascular rings:

With the help of the instructors, familiarize yourself with the BioPac recording system and WPI Myobath II system. To calibrate the BioPac AcqKnowledge recording software, hang weights of specific mass from the tension transducer and set the zero and ca.1000 mg tension. Hang an intermediate weight to check the calibration.

Working with the instructors, dissect the ventral aorta out of either the pithed dogfish shark or pithed eel and place them in cold fish Ringer's solution. Prepare two, 1-2 mm cross-sectional rings of tissue from a single vessel and gently rub the inside of one with a roughened PE tubing to remove the endothelial cells. Groups 2 and 3 will remove the endothelium from both rings from each species. Mount the paired rings in separate Myobath chambers (bubbled with 1% CO_2/99% O_2) at an initial tension of 500 mg for the shark and 100 mg for the eel.

Allow the mounted vascular ring to reach a consistent tension near to these tensions. This may take 30-60 minutes. During this time, make sure you are familiar with the experimental equipment and protocols for this series.

To produce a concentration-response (which is not a dose-response, why?) curve, cumulatively add sufficient agonist to change the experimental bath concentration from zero to the range of 10^{-9} to 10^{-4} M, and record the time course of the tension change to a new, stable value. This might take two hours for each ring. It is important to reach a tension where the last 2-3 additions produce little change. For the first group, after the last ACh addition, add sufficient sodium nitroprusside (SNP; NO donor) to produce a bath concentration of 10^{-4} M. and record the change in tension change. For the third group, preconstrict the rings with 10^{-5} M ET-1 before adding the NPs. Repeat the experiment at least two times during the course of the day to give you at least three data points for each species at each agonist concentration.

You will have plenty of time to read the assigned literature, and plan for the data analysis and your talk, while you wait for the tension to reach a plateau after each ACh addition.

RELEVANT LITERATURE

EDRF

***Furchgott, R. F. and Zawadzki, J. V.** (1980). The obligatory role of the endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376.
Furchgott, R. F. (1996). The discovery of endothelium-derived relaxing factor and its importance in the identification of nitric oxide. *Jama* **276**, 1186-1188.
Furchgott, R. F. (1999). Endothelium-derived relaxing factor: Discovery, early studies, and identifcation as nitric oxide (Nobel lecture). *Angew Chem (Engl)* **38**, 1870-1880.
***Olson, K. R. and Villa, J.** (1991). Evidence against nonprostanoid endothelium-derived relaxing factor(s) in trout vessels. *Am J Physiol Regul Integr Comp Physiol* **260**, R925-R933.
***Evans, D. H. and Gunderson, M. P.** (1998). A prostaglandin, not NO, mediates endothelium-dependent dilation in ventral aorta of shark (Squalus acanthias). *Am J Physiol* **274**, R1050-7.
Evans, D. H. (2001). Vasoactive receptors in abdominal blood vessels of the dogfish shark, *Squalus acanthias*. *Physiol. Biochem. Zool.* **74**, 120-126.
***Feletou, M., Tang, E. H. and Vanhoutte, P. M.** (2008). Nitric oxide the gatekeeper of endothelial vasomotor control. *Front Biosci* **13**, 4198-4217.

Endothelin

***Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T.** (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415.
Masaki, T. (1993). Endothelins: Homeostatic and compensatory actions in the circulatory and endocrine systems. *Endocr Rev* **14**, 256-268.
Olson, K. R., Duff, D. W., Farrell, A. P., Keen, J., Kellogg, M. D., Kullman, D. and Villa, J. (1991). Cardiovascular effects of endothelin in trout. *Am J Physiol Heart Circ Physiol* **260**, H1214-H1223.
***Evans, D. H. and Harrie, A. C.** (2001). Vasoactivity of the ventral aorta of the American eel (*Anguilla rostrata*), Atlantic hagfish (*Myxine glutinosa*), and sea lamprey (*Petromyzon marinus*). *J Exp Zool* **289**, 273-84.
Evans, D. H., Rose, R. E., Roeser, J. M. and Stidham, J. D. (2004). NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. *Am J Physiol Regul Integr Comp Physiol* **286**, R560-R568.
Hyndman, K. A. and Evans, D. H. (2007). Endothelin and endothelin converting

enzyme-1 in the fish gill: Evolutionary and physiological perspectives. *J Exp Biol* **210**, 4286-4297.
***Webb, D. J.** (1997). Endothelin: From molecule to man. *Br J Clin Pharmacol* **44**, 9-20.
***Granger, J. P.** (2003). Endothelin. *Am J Physiol Regul Integr Comp Physiol* **285**, R298-R301.

Natriuretic Peptides

DeBold, A. J., Borenstein, H. B., Veress, A. T. and Sonnenberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* **39**, 89-94.
***DeBold, A. J., Kuroski, de, B. M., Boer, P. H., Dubé, G., Mangat, H. and Johnson, F.** (1991). A decade of atrial natriuretic factor research. *Can J Physiol Pharmacol* **69**, 1480-1485.
Olson, K. R. and Meisheri, K. D. (1989). Effects of atrial natriuretic factor on isolated arteries and perfused organs of trout. *Am. J. Physiol.* **256**, R10-R18.
Evans, D. H., Toop, T., Donald, J. and Forrest, J. N., Jr. (1993). C-type natriuretic peptides are potent dilators of shark vascular smooth muscle. *J Exp Zool* **265**, 84-87.
Donald, J. A., Toop, T. and Evans, D. H. (1994). Localization and analysis of natriuretic peptide receptors in the gills of the toadfish, *Opsanus beta* (teleostei). *Am J Physiol Regul Integr Comp Physiol* **267**, R1437-R1444.
***Evans, D. H. and Harrie, A. C.** (2001). Vasoactivity of the ventral aorta of the American eel (*Anguilla rostrata*), Atlantic hagfish (*Myxine glutinosa*), and sea lamprey (*Petromyzon marinus*). *J Exp Zool* **289**, 273-84.
Tsukada, T. and Takei, Y. (2006). Integrative approach to osmoregulatory action of atrial natriuretic peptide in seawater eels. *Gen Comp Endocrinol* **147**, 31-38.
***Rubattu, S., Sciarretta, S., Valenti, V., Stanzione, R. and Volpe, M.** (2008). Natriuretic peptides: an update on bioactivity, potential therapeutic use, and implication in cardiovascular diseases. *Am J Hypertens* **21**, 733-41.

General

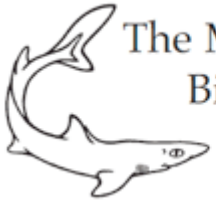
Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Revs.* **85**, 97-177.
Tavares, M., Rezlan, E., Vostroknoutova, I., Khouadja, H. and Mebazaa, A. (2008). New pharmacologic therapies for acute heart failure. *Crit Care Med* **36**, S112-20.
***Barry, S. P., Davidson, S. M. and Townsend, P. A.** (2008). Molecular regulation of cardiac hypertrophy. *Int J Biochem Cell Biol*. (in press).
***Bagnato, A. and Rosano, L.** (2008). The endothelin axis in cancer. *Int J Biochem Cell Biol* **40**, 1443-1451.

*pdfs will be supplied

BIDMC Course on Comparative Physiology

Cardiac Module

Beth Israel Deaconess Medical Center



The Mount Desert Island Biological Laboratory

Cardiac Module: *Squalus acanthias*

Goals:

The goals of the *Squalus* Cardiac Module are geared towards understanding the Starling Principle of the heart and appreciating the hormonal response of the heart to changes in preload and afterload. By way of a simple experimental design, participants will have the opportunity to change the working conditions of the heart and see how this alters the cardiac function. This has clinical applicability particularly in thinking about how to care for patients with cardiogenic shock and those who demonstrate septic physiology with impaired cardiac function. In brief, we hope that participants will achieve the following objectives:

1. Mechanical properties: Explore the relationship between preload and afterload on cardiac output.
2. Hormonal properties: Explore the relationship between preload (atrial stretch) and secretion of cardiac natriuretic peptide.
3. Pharmacologic properties: Explore the relationship between pharmacologic augmentation of contractility and cardiac output.

Readings:

Katz AM. “Ernest Henry Starling, His Predecessors, and the 'Law of the Heart.'" Circulation 2002;106:2986-2992.

Levin ER, Gardner DG, Samson WK (ed. Epstein FH). “Natriuretic Peptides.” NEJM 1998;339(5):321-328.

Solomon R, Bernstein A, Solomon G, Silva P, Epstein FH. “Atriopeptin release from the isolated perfused heart of *Squalus acanthias*: the effects of pressure and chloride concentration.” The Bulletin: MDI Biologic Laboratory 1990;29:82-83.

Woo S and Morad M. “Bimodal regulation of Na/Ca exchanger by B-adrenergic signaling pathway in shark ventricular myocytes.” PNAS 2001;98(4):2023-2028.

Protocol:

Materials:

Shark Heart
1-scalpel
1-razor blade
1-surgery board
1-pithing wire
4-0 silk
Scissors
Forceps
Stop watch
Graduated cylinders (10 ml, 25 ml, 50 ml)
Shark's Ringer's perfusate with albumin
Dobutamine
Heparin

Methods:

1. The isolated perfused shark heart.
    a. Students will harvest the heart from live, pithed dogfish sharks and prepare it for in vitro perfusion.
    b. Inputs:
        i. In the first module: Preload and afterload will be regulated by the height of the perfusate reservoir (preload) and resistance through the conus arteriosus (afterload). Atrial Natriuretic peptide will be measured from the efflux from the heart
        ii. In the second module: Dopamine will be infused at increasing doses to the shark heart
        iii. In the last module: The efflux from the heart before and after preload, afterload and pharmacological challenge will be tested on the rectal gland. The outflow from the heart will perfuse the gland and the efflux will return to the heart. This module will be in combination with the rectal gland module to understand the renal-cardiac interaction.
    c. Outputs:
        i. Cardiac output (min/min) will be recorded for each experimental period using collection of perfusate in a calibrated cylinder for a 60 second time period.
        ii. Arterial pressure measured continuously.
        iii. Natriuretic peptide levels measured from perfusate.
        iv.
2. Experimental periods
    a. Each period will last 30 to 60 minutes and be preceded by an exchange of fresh perfusate.

i. Baseline conditions: preload 2-3 mmHg, afterload 15 mmHg.
ii. Preload conditions mimicking volume expansion: preload 6-7 mmHg, afterload 15 mmHg.
iii. Afterload conditions mimicking essential hypertension: preload 2-3 mmHg, afterload 20-25 mmHg.
iv. Pharmacological challenge: Dopamine infusion at 1 nM, 1 uM, 1 mM concentrations

b. Protocol
   i. Control group: Three periods all at baseline conditions.
   ii. Experimental group 1: Baseline, increased preload, increased afterload, atrial stretch, CO, arterial pressure and ANP measurement.
   iii. Experimental group 2: Baseline, dopamine infusion, CO, arterial pressure and ANP measurement
   iv. Experimental group 3: Baseline, increase preload, increased afterload, dopamine infusion and test of ANP on the rectal gland.

Clinical Questions and Correlation:

Module 1 will focus on the basal preload/afterload setting and apply the concept of changing contractility by mechanical stretch using a balloon in the atria.

1. How do alterations in preload affect cardiac output?
2. How do changes in preload alter the measured arterial pressure?
3. What effects do changes in afterload have on cardiac output?
4. What is the explanation for these observations?
5. How does atrial stretch (preload) influence the secretion of cardiac natriuretic peptides?
6. Do changes in afterload alter the secretion of cardiac natriuretic peptides?

Clinical correlates: How does this apply to the management of patients who are in cardiogenic shock or who have reduced cardiac function? What clinical conditions mimic the affects of afterload on the heart and how can we augment or change afterload to improve cardiac function?

Module 2 will apply the changes in contractility by pharmacological modification of heart rate and cell force identifying the compensatory mechanisms in place physiologically as well as the effect of pharmacological management of patients in acute heart failure

1. What type of effect does dopamine have on preload and afterload?
2. What type of effect does dopamine have on secretion of cardiac natriuretic peptides?
3. What type of effect does dopamine have on cardiac output?

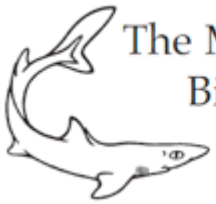
Module 3 will combine the renal and cardiac module identifying the interplay between organs in the management of cardiac function and in the multi organ syndrome of heart failure.

1. How do natriuretic peptides regulate blood volume in sharks and in humans?
2. Do changes in the level of natriuretic peptides correlate with the degree of cardiac function?

BIDMC Course on Comparative Physiology

Hematology Module

Beth Israel Deaconess Medical Center

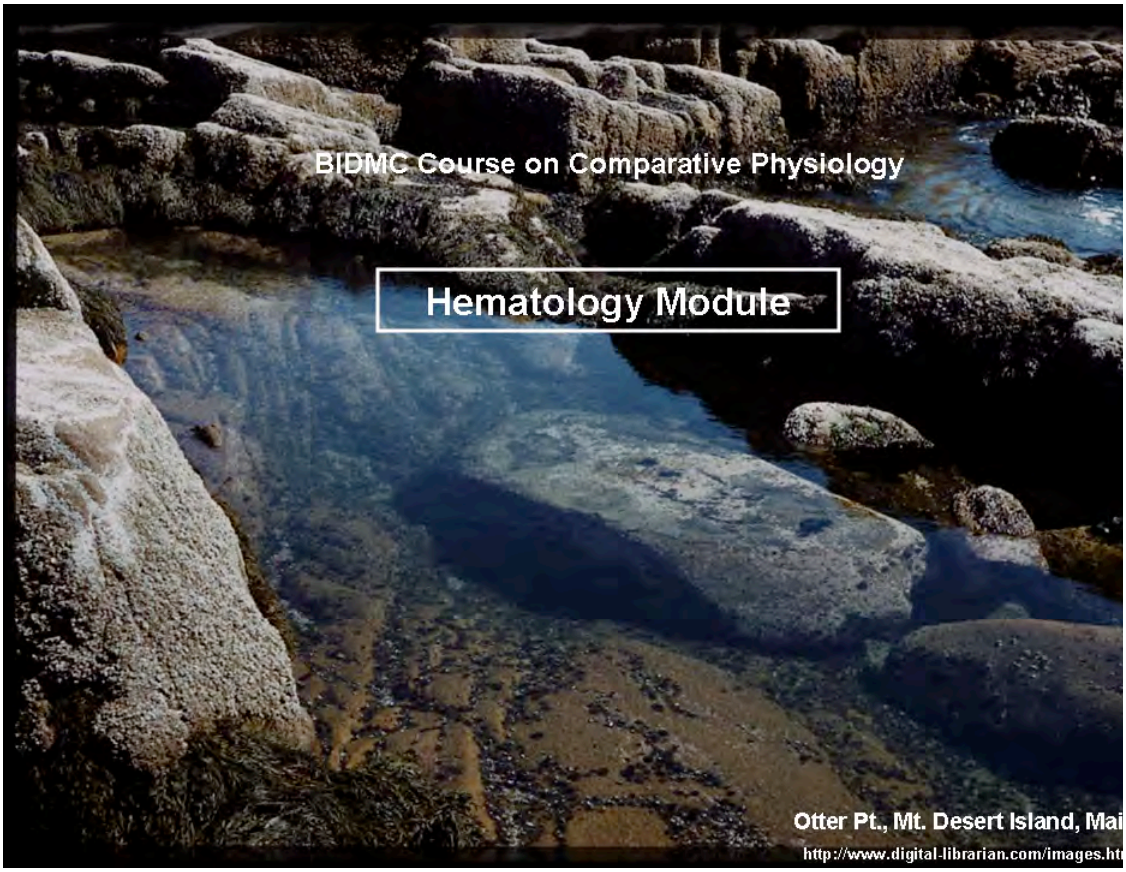


The Mount Desert Island Biological Laboratory

BIDMC Course on Comparative Physiology

HEMATOLOGY MODULE

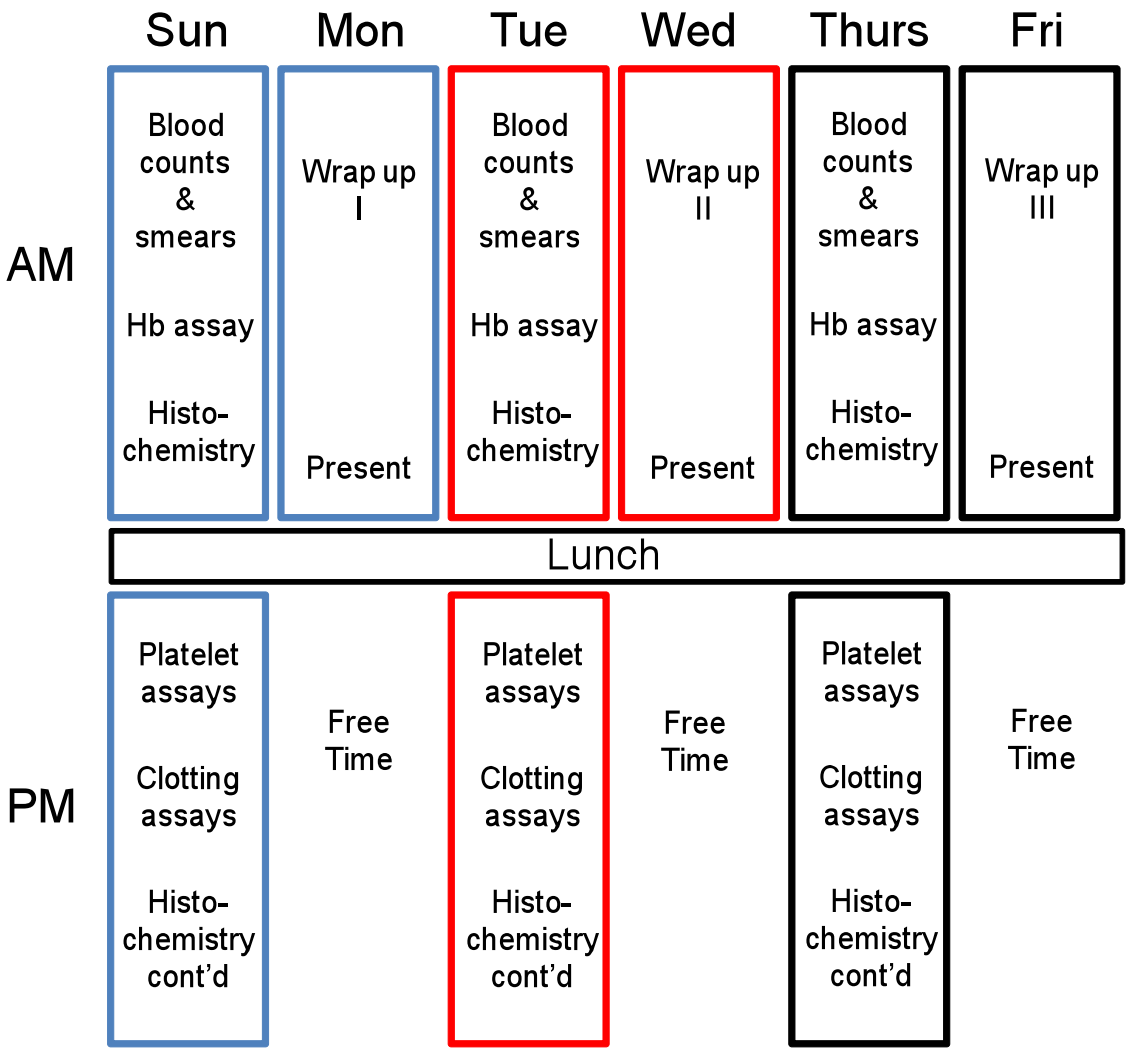
William Aird
Alicia Clark
Marianne Grant
Alan Rigby



GOALS AND SCHEDULE

The overall goal of this module is to underscore basic principles of hematology, including red cell quantitation ,morphology, and hemoglobin content; platelet function; and hemostasis and blood coagulation. The module consists of a number of core specific aims, which are common to all three sessions (S/M, T/W, Th/F), and a number of specific aims that will vary between the three sessions. The core aims are the following:

1. Calculate and measure red cell indices in multiple species
2. Make and interpret peripheral smears in multiple species
3. Test platelet aggregation in multiple species
4. Examine clotting times and the expression patterns of clotting/coagulation proteins
5. Clone of genes for blood hemostasis and coagulation proteins from Hagfish



BACKGROUND

Comparative physiology

Single cell organisms obtain their oxygen through simple diffusion, a process that is defined by Fick's law (the flow of oxygen is directly proportional to the pressure difference and surface area, and inversely proportional to distance oxygen must travel). In multicellular organisms, the cardiovascular system provides a means of overcoming the time-distance constraints of diffusion.

As a general rule, the body plan of most multicellular organisms can be simplified according to the scheme shown in **Fig. 1** (there are some interesting exceptions to this body plan, including insects). There are four basic elements to the scheme: 1) convection or bulk flow of oxygen from the environment to a highly vascularized surface, 2) oxygen diffusion from environment to blood, 3) convection or bulk flow to the various tissues of the body, and 4) diffusion across into the mitochondrial "sink" of each and every cell. Note that the laws of nature have not been defied: oxygen transport is still critically dependent on simple diffusion, both at the lung-blood interface and the blood-tissue interface.

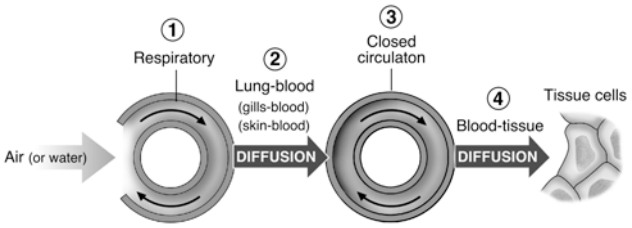


Fig. 1. **Pathway for oxygen in multicellular Organisms**. This schematic simplifies oxygen transport according to 4 steps: 1) Convection of oxygenated air or water from environment to a highly vascularized surface (skin, gills, or lungs), 2) diffusion of oxygen across the gas exchanger into the blood, 3) convection of oxygenated blood around to the various tissues of the body, and 4) diffusion of oxygen to the individual cells of the tissues.

Oxygen is poorly soluble in water or plasma. Therefore, even in simple multicellular organisms, oxygen delivery is aided by the presence of a respiratory pigment, a molecule that essentially acts like a magnet to attract and carry oxygen. In invertebrates, the respiratory pigment (usually hemocyanin, but sometimes hemoglobin) circulates freely in solution. This observation provides the rationale for developing hemoglobin substitutes in transfusion medicine. In vertebrates, the hemoglobin is packaged inside red blood cells, where it is protected from oxidative stress of the environment and where oxygen binding may be finely regulated through a series of allosteric and cooperative interactions.

Non-mammalian vertebrates (fish, amphibians, reptiles and birds) contain nucleated red cells (**Fig. 2**) [their platelets are also nucleated, and are termed thrombocytes]. In fact, the anulceate red blood cell is unique to mammals. It is interesting to speculate why the nucleus may have been "discarded" during recent evolution. There are many possible

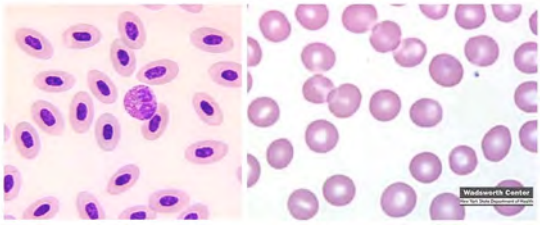


Fig. 2. **Red blood cells in non-mammalian vertebrates (left) and mammals (right).**

explanations. Perhaps the most compelling is that anucleate cells lack mitochondria and oxidative phosphorylation, and therefore do not consume oxygen. In this way, the mammalian red blood cell avoids the conflict of interest of being both a carrier and consumer of oxygen. Countering this argument is the observation that hummingbirds, which transport their oxygen in nucleated red cells, have a higher metabolic rate compared with most mammals.

Whether from a 70-kg human, a 2-g shrew or a 150-ton blue whale, the mammalian red blood cell is remarkably similar in size and shape (there are some interesting exceptions which will not be discussed here) (**Fig. 3**). The high degree of invariance in cell size and shape is consistent with the notion that selection - acting upon the mammalian red blood cell - has led to the optimization of the variables in Fick's equation. For example, the biconcave shape provides a high surface area-to-volume ratio and a short distance for oxygen to travel.

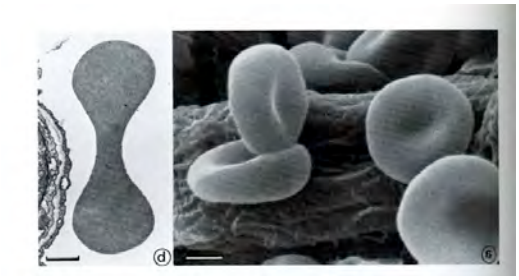


Fig. 3. **Biconcave shape of mammalian RBC.**

During evolution, a solution to one problem tends to beget a new set of problems. As one example, the development of a cardiovascular system, while providing a means to overcome the time-distance constraints of diffusion (and thereby paving the way for the evolution of large animals), resulted in a highly pressurized system that is at risk for rupture and/or leakage, with potentially two life-threatening consequences: 1) exsanguination or loss of blood from the interior to the exterior (hence, the formation of the coagulation mechanism, consisting of "sticky" cells and a protein gel), and 2) the entry and dissemination of pathogens from the exterior to the interior (hence, the formation of the innate immune response, also composed of cells and a protein gel).

During this course, we will expand considerably on the evolutionary and comparative principles related to red blood cells, platelets and coagulation.

Red cell quantitation and morphology

Hemoglobin (Hb) values are expressed as grams of Hb per liter of whole blood. The hematocrit represents the proportion of whole blood that is occupied by red blood cells. The Hb, Hct and red blood cell count can be used to calculate the so-called "red cell indices", including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

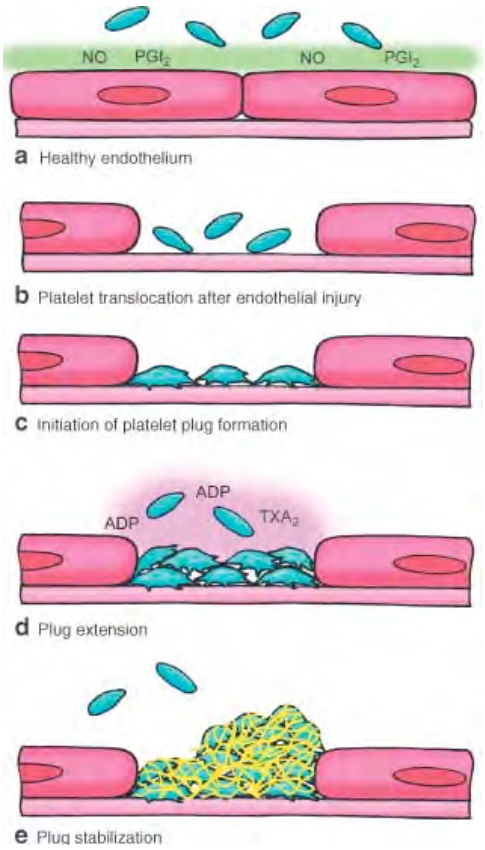
MCV (L) = Hct x red cell count (number/L)
MCH (g) = Hb (g/L) ÷ red cell count (number/L)
MCHC (g/L) = Hb (g/L)/Hct

In the clinical lab, automated CBC machines measure the Hb, red cell number and MCV and use these values to calculate Hct, MCH and MCHC. In the field (for example, in rural areas), Hct, red cell count, and Hb are measured, whereas MCV, MCH and MCHC are derived.

Examination of the peripheral smear is an essential component of the work up of patients with hematological abnormalities, such as anemia. The most common stain is Wright-Geimsa, which you will use in this exercise. In the clinical setting, the peripheral smear is examined at low power for evidence of agglutination or rouleaux. At high power, red cell morphology is examined for size (average size and range in size), central pallor (increased central pallor is called hypochromia), abnormal shapes (the presence of abnormal shapes is called poikilocytosis), and inclusions. Peripheral smears are also surveyed for platelet and leukocyte number and morphology.

Platelets

Injury to the endothelial layer exposes collagen and other components of the subendothelial connective tissue. von Willebrand factor (vWF) within the subendothelial connective tissue forms a bridge between platelets (GPIb) and collagen, resulting in platelets adhesion to the matrix. Several events including binding of vWF to GPIb- result in outside-to-inside signal transduction and subsequent platelet activation. Subsequent inside-out signaling results in conformational change of the GPIIb-IIIa receptor. Activated GPIIb-IIIa is essential for platelet aggregation (and thus thrombus formation). Activated platelets undergo cytoskeletal rearrangement and display shape change (discoid to spherical), with extension of branch-like filopodia. GPIIb-IIIa can then bind vWF, fibrinogen, fibronectin, vitronectin and thrombospondin, providing further important sites of anchorage for the spreading platelet.



Platelet plugs involve the following steps:

1. Initiation phase (adhesion) - platelet monolayer over the subendothelium
2. Extension phase (aggregation) - platelet recruitment following stimulation by locally produced platelet agonists, such as thrombin and mediators released directly from platelets (e.g. adenosine diphosphate [ADP]). Mediated largely by fibrinogen, which bridges clustered GPIIb-IIIa surface receptors.
3. Perpetuation phase - fibrinogen is converted to fibrin by thrombin, further stabilizing the platelet plug, preventing premature disaggregation.

Platelet aggregometry involves measuring platelet-to-platelet aggregation in response to an agonist in platelet-rich plasma by turbidometry/optical density (as platelets aggregate,

more light can pass through the specimen) or in whole blood by electrical impedance. Agonists include ADP, epinephrine, collagen, ristocetin, and arachidonic acid. The fundamental advantage of platelet aggregometry is that it measures, albeit in an ex vivo system, the most important function of platelets: their aggregation with one another in a GPIIb/IIIa–dependent manner. The major disadvantages to platelet aggregometry as a clinical test of platelet function, include poor reproducibility, high sample volume, requirement for sample preparation, length of assay time, the requirement of a skilled technician, and expense.

The most common cause of abnormal platelet aggregometry test is medications, particularly aspirin (in arachidonate-mediated aggregation is markedly decreased or absent, along with other aggregation tracings). Other medications that interfere with platelet aggregation in this assay include ticlopidine, clopidogrel, and abciximab, are known to impair platelet aggregation. Other acquired causes of impaired platelet aggregation include uremia and myeloproliferative disorders. Hereditary platelet disorder are much less common, and include platelet storage pool disorders (which may be associated with decreased responses to epinephrine and/or ADP) and Glanzmann thrombasthenia (GPIIb/IIIa deficiency). Since GPIIb/IIIa mediates platelet aggregation via fibrinogen binding, Glanzmann thrombasthenia is associated with reduced aggregation with all agonists (ADP, collagen, epinephrine, arachidonate) except ristocetin.

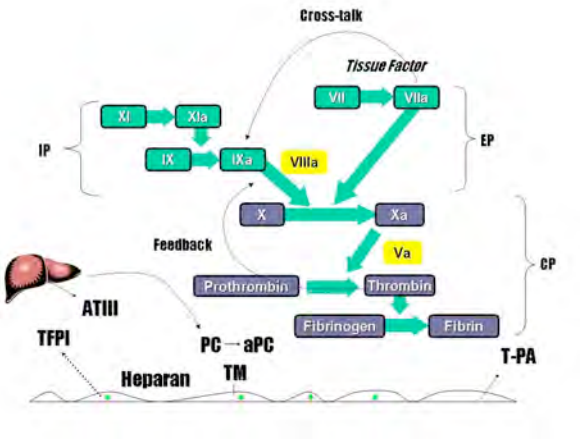


Fig. 4. **The coagulation pathway**. The clotting cascade consists of an extrinsic pathway (EP; tissue factor, factor VII), an intrinsic pathway (IP; factors XI, IX, VIII), and a common pathway (CP; factors X, V, (pro)thrombin and fibrinogen). Factors VII, XI, IX, X, II (thrombin) are serine proteases; factors V and VIII are cofactors; fibrinogen is a structural protein. Shown are the four major classes of natural anticoagulants: antithrombin III (ATIII)-heparan (which inhibits the serine proteases of the clotting cascade), protein C (PC)/protein S (not shown) and thrombomodulin (TM) (which inhibits the cofactors of the clotting cascade), tissue factor pathway inhibitor (TFPI) (which inhibits the extrinsic pathway) and the fibrinolytic systems (plasmin degrades fibrin). The liver and endothelium (the cell lining at bottom) both contribute to the synthesis and release of hemostatic factors. Note that factor XII is not included in the scheme. This factor, which can activate factor XI in vitro, is important to consider when interpreting results of coagulation assays. However, it is not involved in mediating in vivo hemostasis. Two key links connect the extrinsic and common pathways with the intrinsic pathway. First, factor VIIa activates factor IX (cross talk). Second, thrombin activates factors XI and VIII (feedback). t-PA; tissue-type plasminogen activator. The activated form of the serine protease is indicated by the suffix “a”.

Coagulation

Hemostasis is often divided into primary and secondary limbs. Primary hemostasis, which was discussed in the previous section, involves the activation, adhesion, and aggregation of platelets. Secondary hemostasis, which culminates in fibrin formation, consists of a series of linked reactions in which a serine protease once activated is capable of activating its downstream substrate. Primary and secondary hemostasis are integrally linked in both time and space.

When approaching the blood clotting mechanism, several important themes emerge. First, the clotting cascade ultimately results in conversion of fibrinogen to fibrin, a process that is mediated by the serine protease thrombin. Second, fibrin not only strengthens aggregates of platelets, but may also contribute to the host defense by “walling off” pathogens and facilitating their engulfment by white blood cells. Third, blood coagulation is always initiated by the extrinsic pathway (via tissue factor (TF)-mediated activation of factor VII), and is amplified through the intrinsic pathway (by virtue of cross talk and feedback) (**Fig. 4**). Fourth, every procoagulant step is balanced by one or more natural anticoagulants. For example, tissue factor pathway inhibitor (TFPI) neutralizes the extrinsic pathway; heparan acts as a cofactor for antithrombin III (ATIII), which serves to inhibit every serine protease in the cascade (most notably factors Xa and thrombin); activated protein C functions with its cofactor protein S to inactivate the cofactors of the procoagulant response (FVa and FVIIIa); and plasmin degrades cross-linked fibrin. This latter process, termed fibrinolysis, results in generation of d-dimers.

In the final analysis, hemostasis represents a finely tuned balance between procoagulant and anticoagulant forces. The procoagulant side includes platelets and soluble clotting factors. The anticoagulant side includes blood flow, maintenance of anticoagulant phospholipid cell surface, and the expression of specific anticoagulant proteins.

METHODS AND RATIONALE

Red cell count

Reagents and equipment required

Fish blood collected in EDTA tube (purple top)
Hemocytometer
Microscope

Methods

This measurement is made with a microscope and a specially ruled chamber (hemocytometer). The counting chamber and cover slip must be clean and dry. The polished surfaces and rulings are easily scratched, so lens paper moistened with the above solutions is used for cleaning. The entire chamber and cover slip are then lightly dried with lens paper. **Fig. 6** shows the rulings which are inscribed on the counting chamber. The smallest squares in the large center square (where red cells are counted) have an area of 1/400 mm and are arranged in groups of 16. Each group of 16 squares is set off from the others by triple lines. The middle line is the one which actually defines the area of the squares adjacent to such a triple line. The inner line, therefore, is included within the area of the square. Erythrocytes in five of the 25 groups of 16 small squares are counted. These groups are the four corner groups and the one in the center. Count all the cells which touch the upper and left boundary lines (middle of the three lines) of the squares, but do not count those which touch the lower and the right boundary lines. A period of 2 to 3 minutes after filling allows the red cells to settle to the bottom of the chamber so that they will be in focus. Examine the chamber at 100X magnification for evenness of the red blood cell distribution. Turn the 40X objective in place; focus and count the cells in the designated squares.

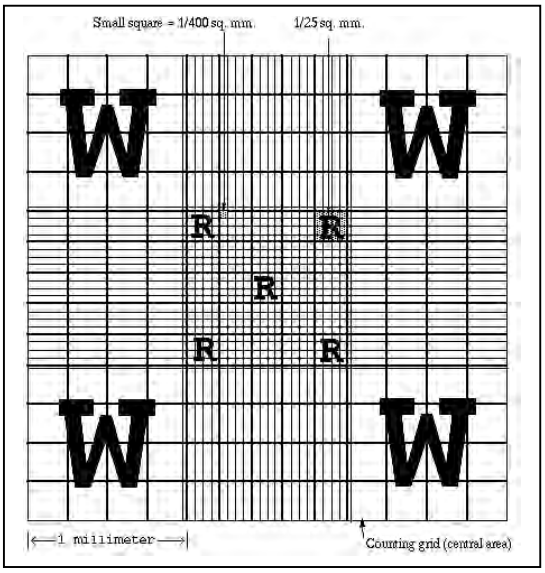


Fig. 6. **Hemocytometer.**

Hematocrit

Reagents and equipment required

Fish blood collected in EDTA tube (purple top)
Capillary tubes (with sealed end)
Micro-hematocrit centrifuge
Micro-hematocrit reader

Methods

Hematocrit is the percent volume of whole blood occupied by red blood cells and is determined by centrifuging blood in special (hematocrit) capillary tubes. Touch the end of a heparinized, capillary hematocrit tube to the edge of the blood sample and allow the tube to fill three-fourths full. Tilt the tube to leave both ends free of blood. Seal one end of the tube with clay furnished with the tubes. Centrifuge for 5 min. at 10,000 RPM in a micro-hematocrit centrifuge, or for 30 min. at high speed in a regular single head centrifuge. After centrifugation, determine the hematocrit by measuring both the total height of blood and plasma and the height of the blood cell column (ignore the white buffy layer) to the nearest 1/2 mm. The hematocrit is calculated by: Hct (%) = [Height of Packed Red Cells (mm) x 100] / [Height of Packed Red Cells and Plasma (mm)].

Hemoglobin

Reagents and equipment required

Fish blood collected in EDTA tube (purple top)
Drabkin's reagent (Sigma)
Hemoglobin for preparation of standard curve (bovine)
Cuvettes
Test tubes
Spectrophotometer (Smith Equipment Room)

Methods

Hemoglobin content of whole blood is determined by photometric methods and recorded as gm/dL (or g/L). Prepare a cyanomethemoglobin Standard Solution containing 90 mg in 500 µl. Then prepare dilute standard by diluting 40 µl of the concentrated Standard Solution into 10 ml Drabkin's solution. Prepare working standards indicated below.

Tube #	Dilute CyanoHb standard (ml)	Drabkin's solution (ml)	CyanoHb concentration (µg/ml)
1	0.0	3.0	0
2	1.0	2.0	240
3	2.0	1.0	480
4	3.0	0.0	720

Add 20 µl of blood to 5.0 ml of Drabkin's solution. Rinse out the pipette twice with the Drabkin's reagent. Mix the contents using the vortex mixer. Incubate for at least 15 minutes at room temperature. Read and record absorbance vs. blank at 540 nm using a plastic cuvette (these hold 1 ml). Using the standard Hb curve, determine the Hb concentration of your blood.

Blood smear

Reagents and equipment required

Fish blood collected in EDTA tube (purple top)
Slides
Wright's staining solution (Sigma)
Giemsa staining solution (Sigma)
Microscope

Methods

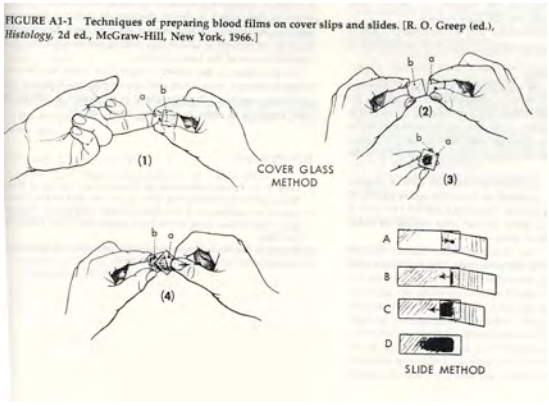


Fig. 7. **Peripheral smear**

To prepare the smear place a small drop of blood on the surface of a clean microscope slide near the end (**Fig. 7**); using a second microscope slide as a spreader held at 30-40 degrees, touch its end to the edge of the blood drop and push it toward the opposite end of the slide - the blood will be drawn into the acute intersection of the slides and will be "pulled" across the slide (see illustration). Allow the smear to air dry for about 5 minutes. To stain the smears place the smears in Wright's stain for 3 minutes, transfer to water or phosphate buffer (pH 6.2) for 3 minutes and then briefly rinse with water. Place the slide in a vertical support and allow to air dry.

Platelet aggregation

Reagents and equipment required

Chrono-Log Whole Blood Aggregometer

Principle

We will be using a whole blood aggregometer which measure aggregation directly in whole blood (instead of platelet rich plasma [PRP]). Whole blood represents a more physiological milieu than PRP, and the assays require less blood (which is a limiting factor in our fish experiments). The system employs electrical impedance to measure platelet aggregation. A diluted specimen of whole blood is placed in the sample cuvette which is then placed in the instrument reaction well. The sample cuvette included a disposable electrode. The platelets in the sample adhere to the two fine metal pins on the electrode, forming a uniform monolayer of platelets on the pins. A small voltage difference is applied across the two pins, and the impedance caused by the platelets coating the pins is measured. In the absence of an aggregating agent, or agonist, the interactions between the platelets and the electrodes stabilize and the impedance between the two electrodes becomes constant, producing a stable baseline. When an agonist is added, platelets in the specimen are activated and begin to aggregate. The coating of platelets thickens on the pins over the next few minutes with a corresponding increase in the electrical impedance between the electrode wires. This change in impedance is directly proportional to the extent of platelet aggregation and is indicated on the digital display, in ohms, after 6 minutes.

Methods

To be discussed during the course

Immunohistochemistry

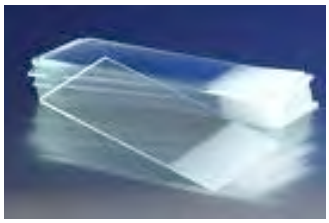
Reagents and equipment required

Sections of tissue cut from OCT frozen blocks mounted on Superfrost slides
Appropriate species specific primary antibody solution
Biotinylated-secondary antibody solution
HRP-conjugated tertiary antibody solution
DAB staining/detection reagents
Hematoxylin stain
Microscope

Methods

Immunohistochemistry or IHC refers to the process of localizing antigens (e.g. proteins) in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualizing an antibody-antigen interaction can be accomplished in a number of ways.

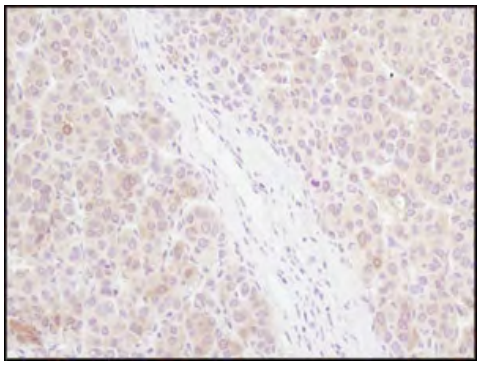
Cryostat-microtome sectioning



A cryostat will be used to cut frozen organ tissue for frozen section histological slides. The cryostat is essentially an ultrafine "deli-slicer", called a microtome, placed in a freezer. Specimens will be frozen in cutting medium and cut sections of frozen tissue will be mounted on clear glass slides, where they will melt and adhere. The glass slides and specimens will be dried and stained.

Immunohistochemical staining

We will use an indirect staining method involving an unlabeled primary antibody (first layer) which reacts with tissue antigen, a labeled secondary antibody (second layer) which reacts with the primary antibody, and a labeled tertiary antibody (third layer) which reacts with the secondary antibody. This method is more sensitive due to signal amplification through several secondary/tertiary antibody reactions with different antigenic sites on the primary antibody and interaction between the secondary and tertiary antibodies. We will use a biotinylated secondary antibody and a tertiary antibody coupled with strepavidin-horseradish peroxidase. This is reacted with 3,3'-Diaminobenzidine (DAB) to produce a brown staining wherever primary and secondary antibodies are attached in a process known as DAB staining. Diluted filtered Hematoxylin can also be used in the staining of cells for contrast staining and produces a blue color.



Coagulation Assays

Reagents and equipment required

Species specific plasma (citrated)
Species specific clotting initiation fraction (tissue factor preparations/thromboplastin)
Temperature regulated water bath
25mM $CaCl_2$ solution
Borosilicate glass tubes

Methods

Blood collection

A blood samples will be collected in tubes on ice, the volume measured, and 1/6 vol of ice-cold citrated (3.8%) HEPES buffer will be added and mixed to chelate any blood calcium and thus inhibit coagulation. Then the upper plasma layer will be removed from the red blood cells by centrifugation at 2000 x *g* for 4 min.

Prothrombin time (PT)

The prothrombin time (PT) is an assay designed to screen for activities of fibrinogen, prothrombin, and factors V, VII, and X and thus measures activities of the extrinsic pathway of coagulation. When any of these factors is deficient then the PT is prolonged. The PT will be measured using plasma after the blood cells are removed. Excess calcium will be added to the plasma to initiate coagulation. Clotting times will be determined in glass tubes in a temperature controlled water bath by visual inspection of the formation of the fibrin clot. Clinically PT is used to determine the correct dosage of the warfarin class of anti-coagulation drugs (e.g. Coumadin), for the presence of liver disease or damage, and to evaluate vitamin K status.

Partial thromboplastin time (PTT)

The partial thromboplastin time (PTT) is used to assay for defects in the intrinsic pathway of coagulation. The PTT assay has been modified by the addition of activators that shorten the normal clotting time and this form of the assay is referred to as the activated partial thromboplastin time (aPTT). The assay will evaluate the function of fibrinogen, prothrombin, and factors V, VIII, IX, X, XI, and XII. A defect in any of these factors will result in a prolonged PTT (or aPTT). Clinically, the PTT is a standard assay used to assess the efficacy of heparin anticoagulant therapy. Prolonged PTTs are associated with acquired or congenital bleeding disorders associated with coagulation factor deficiency, vitamin K deficiency, liver disease, DIC, von Willebrand disease, leukemia, hemophilia, and during heparin administration. Clotting times will be determined manually with visual inspection, as above.

Cloning of genes from hagfish

Reagents and equipment required

PCR machine
Agarose gel

Principle

PCR-based Gene Cloning. Gene cloning is the act of making copies of a single gene. Amplified genes are useful in many areas of research and for medical applications such as gene therapy. Selective amplification of genes depends on our ability to perform the following essential procedures.

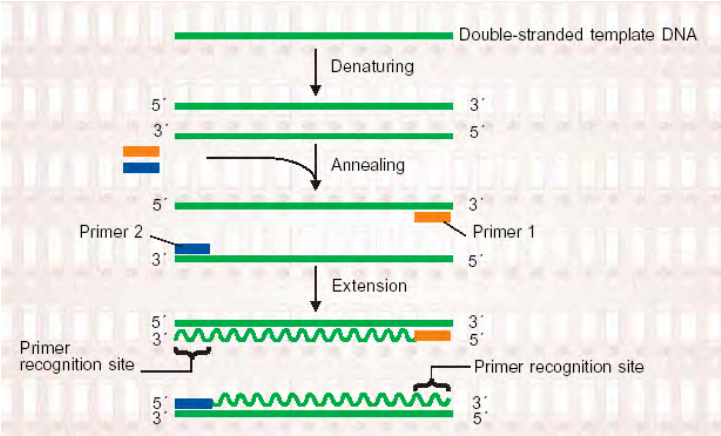
1. PCR Amplification of a Specific Gene. PCR is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality.

PCR procedure uses three important steps in the reaction:

Denaturating: DNA fragments are heated at high temperatures, which reduce the DNA double helix to single strands. These strands become accessible to primers.

Annealing: The reaction mixture is cooled down. Primers anneal to the complementary regions in the DNA template strands, and double strands are formed again between primers and complementary sequences.

Extension: The DNA polymerase synthesizes a complementary strand. The enzyme reads the opposing strand sequence and extends the primers by adding nucleotides in the order in which they can pair. The DNA polymerase, known as 'Taq polymerase', can withstand the high temperatures needed for DNA-strand separation, and can be left in the reaction tube.



The key to PCR amplification is that the cycle of heating and cooling is repeated over and over, stimulating the primers to bind to the original sequences and to newly synthesized sequences. The polymerase enzyme will again extend primer sequences. This cycling of temperatures results in copying and then copying of copies, and so on, leading to an exponential increase in the number of copies of specific sequences. Because

the amount of DNA placed in the tube at the beginning is very small, almost all the DNA at the end of the reaction cycles is copied sequences. The reaction products are separated by gel electrophoresis.

2. Cutting DNA at Precise Locations. The discovery of enzymes known as restriction endonucleases has been essential to protein engineering. These enzymes cut DNA at specific locations based on the nucleotide sequence. Hundreds of different restriction enzymes, capable of cutting DNA at a distinct site, have been isolated from many different strains of bacteria. DNA cut with a restriction enzyme produces many smaller fragments, of varying sizes. These can be separated using gel electrophoresis or chromatography.

3. Joining Two Pieces of DNA. To link two or more individual strands of DNA, to create a longer strand, or close a circular strand that has been cut with restriction enzymes, enzymes called DNA ligases can create covalent bonds between nucleotide chains.

4. Insertion of Amplified Gene into Self-Replicating Vector DNA. Small circular pieces of DNA that are not part of a bacterial genome, but are capable of self-replication, are known as plasmids. Plasmids are often used as “vectors” to transport genes between microorganisms. Once the gene of interest has been amplified and both the gene and plasmid are cut by restriction enzymes, they are ligated together generating what is known as a recombinant DNA.

5. Moving the Recombinant DNA Vector into a Host Cell. The process of transferring plasmids into new host cells is called transformation. This technique requires that the host cells are exposed to a heat-shock, which makes them “competent” or permeable to the plasmid DNA.

6. Method to Select Hosts Expressing Recombinant DNA. Not all cells will take up DNA during transformation. It is essential that there be a method of detecting the ones that do. Generally, plasmids carry genes for antibiotic resistance and transformed cells can be selected based on expression of those genes and their ability to grow on media containing that antibiotic. Additional methods of selection depend on the presence of other reporter proteins such as the x-gal/*lacZ* system, which allows selection based on the color appearance of the cells.

7. Nucleotide Sequencing the Inserted Gene Product. The 'dye-terminator sequencing’ method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP). These dideoxynucleotides are the *chain-terminating* nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand therefore terminates DNA strand extension, resulting in various DNA fragments of varying length. In 'dye-terminator sequencing' each of the four dideoxynucleotide chain terminators is labeled with a different fluorescent dye, each fluorescing at a different wavelength. The

newly synthesized and fluorescently labeled DNA fragments are heat denatured, and separated by size (with a resolution of just one nucleotide) by gel electrophoresis on a denaturing polyacrylamide-urea gel. Each of the four DNA synthesis reactions is run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by fluorescence, and the DNA sequence can be directly read off the gel image by a computer-controlled sequence analyzers.

Methods

Isolate RNA and generate cDNA from hagfish organs. This will have been completed prior to the start of the course. Briefly the protocol was as follows: Liver and gill were removed from a single adult hagfish. RNA was extracted from each, separately. RNA quality and concentration was assessed by spectrophotometry and 1% agarose/formaldehyde gel electrophoresis. First-strand cDNA synthesis was performed with 1 µg of total RNA, oligo(d)T primers, and Superscript II (Invitrogen). The cDNA was aliquoted and stored at -80^{o}C.

Clone prothrombin using degenerate primers. We have already created degenerate primers designed to match conserved regions of the prothrombin protein (we will discuss how we arrived at these sequences). PCR will be carried out with 5 µl of the synthesized cDNA. Cycles The amplified fragments of the expected size will be excised from ethidium bromide-stained agarose gels, purified and subcloned into pDrive cloning vector (Qiagen). The resulting plasmid will be transformed into competent DH5α, and the cloned PCR fragment will be sequenced using an ABI prism 377 automatic sequencer.

QUESTIONS

Red Cells

1. What are the differences in Hb, Hct and red cell indices between species?
2. Which of these variables did you predict to be most constant between species, and why?
3. How do these values impact on oxygen delivery?
4. What are the selective advantages of “packaging” respiratory pigment in cells (as vertebrates do)?
5. What are the selective advantages of anucleate (as distinct from nucleated) red blood cells?
6. We often think of the Hb being 1/3 that of the Hct (e.g. Hct 30% = Hb 10 g/dl). What are some clinical conditions in which this rule is broken?

Platelets

7. What are the selective advantages of anucleate (as distinct from nucleated) platelets?
8. Would you predict that primary hemostasis (cell response) or secondary hemostasis (clottable proteins) are most evolutionarily considered, and why?

Coagulation

9. If you were to identify novel anticoagulants, where in the animal kingdom would you look?
10. What do you believe are the minimum requirements for a clotting cascade in animals (e.g. which procoagulant and/or anticoagulant factors must be present)?

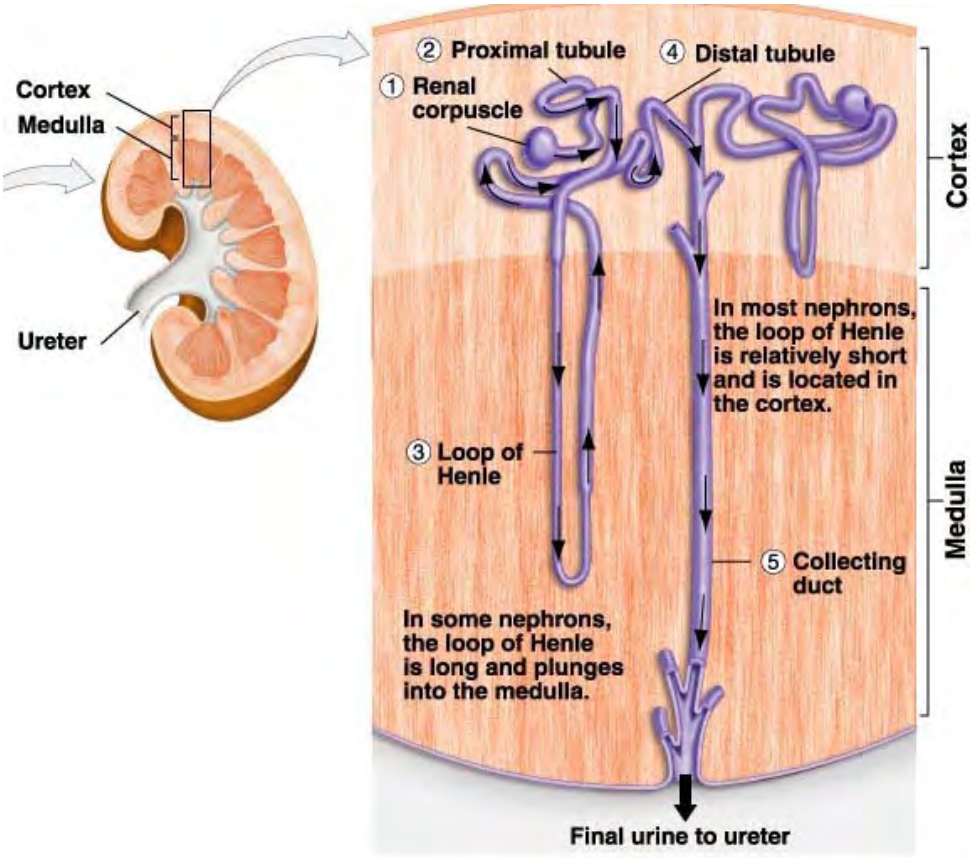
BIDMC Course on Comparative Physiology

Water Module

BIDMC Course on Comparative Physiology

WATER PHYSIOLOGY MODULE

Shani Herzig
Warren Hill
Bryce MacIver
John C. Mathai
Mark Zeidel

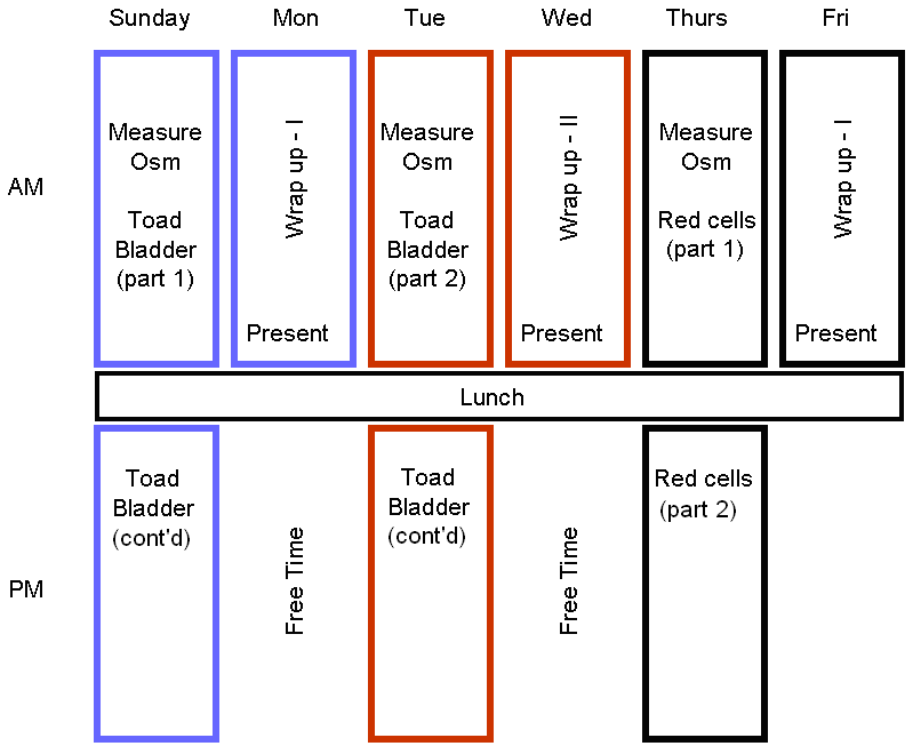


GOALS AND SCHEDULE

The overall goals of this module are to: 1) Gain an insight into the physiological challenges faced by marine organisms in maintaining appropriate water balance; 2) sharpen understanding of the concept of “effective” versus “ineffective” osmols, and the measurement of osmolality in the blood; 3) gain an appreciation of human water homeostasis through the study of membranes containing AQP1 and AQP2 homologs; 4) illustrate disease states in human beings via animal models.

In order to achieve the above goals, we will be observing water flux directly or indirectly across three different types of membranes/systems:

1) Red blood cells
2) Toad bladder
3) Human nephron



BACKGROUND

Osmotic (water) homeostasis

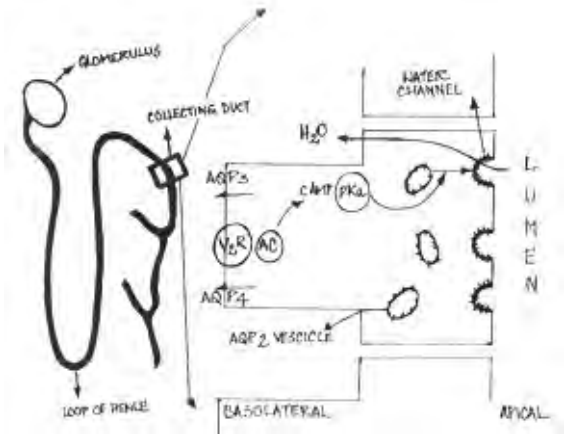
The human urinary system functions to excrete nitrogenous wastes, to balance salt excretion with its absorption, and to maintain the composition of the blood so that osmolality and concentrations of ions such as potassium and calcium remain within narrow ranges. Osmoregulation is elegantly achieved in human beings via a complex interplay of salt and water diffusion/transport in the renal medulla, forming the basis for the famous, (or, infamous), "countercurrent multiplier mechanism." In healthy kidneys, the medullary interstitium is maintained at an extremely high osmolality (1200 mOsm/kg in human, > 6,000 mOsm/kg in the desert rat, *Psammomys*) through the interplay of active salt transport and passive fluxes of water and urea. By inserting or not inserting apical membrane water channels (or aquaporins) in the collecting duct, humans are able to achieve fine regulation and remarkable consistency of tissue osmolality (approximately 300 mOsm/kg).

A great deal of information about the physiologic processes occurring in the human kidney has been gained through study of animals residing in aquatic environments. Such animals face the extremes of osmolality, ranging from fresh water (osmolality far less than 300 mOsm/kg) to sea water (osmolality around 900-1000 mOsm/kg). While some fish have developed the ability to survive in a range of osmolalities - such as the eel and salmon - others lack these mechanisms and are therefore iso-osmotic to their surrounding and thus relegated to only one type of water - such as the hagfish. In the absence of mechanisms to maintain constant osmolality, as in the hagfish, movement to fresh water would lead to catastrophic water gain, while movement to salty sea water would lead to dehydration.

The mechanisms by which fish and other animals are able to maintain their constant osmolality even in the face of extreme osmotic forces will be the focus of the following modules. Given that an understanding of ion flux, osmolar gradients, and hormone-receptor interactions are fundamental to understanding the human kidney, we hope that the following modules will challenge and sharpen your understanding of these same processes as they occur in your own patients.

Anti-diuretic hormone: water flux across the toad bladder

The toad bladder has many of the ion and water transport properties found in the collecting duct of the nephron. Because it was easy to study as a sac or when mounted in an Ussing chamber (to be discussed later), it was the experimental model used to work out the framework of salt and water homeostasis along the nephron. Toad bladders possess a vasopressin-inducible water channel which is a homolog of human aquaporin 2. In response to hormone binding to the



Aquaporin function in the principal cells of the collecting duct

vasopressin receptor on the basolateral side of the collecting duct epithelium (or toad bladder epithelium), intracellular cAMP is increased, resulting in activation of protein kinase A and subsequent phosphorylation of AQP2 in endosomes. Endosomes containing phosphorylated AQP2 then traffic to and fuse with the apical membrane. Under basal conditions, the apical membrane serves as a barrier epithelium, specialized to permit only very slow flux of water across it. Insertion of AQP2 water channels leads to a 100 fold increase in water permeability per unit membrane area.

In humans, provided the remainder of the nephron is functioning properly, ADH is the main hormone responsible for the final concentration of the urine, and thus, maintenance of plasma osmolality. In the presence of high levels of ADH, aquaporins are inserted into the lumenal membrane of the late distal tubule and collecting duct, allowing passive resorption of water along the concentration gradient established by the more proximal urinary diluting segments of the nephron. Under maximal stimulation, urinary osmolality can be as high as 1200 mosm/kg. In the absence of ADH, on the other hand, the cells of the late distal tubule and collecting ducts are impermeable to water, and urinary osmolality may be as low as 50 mosm/kg.

METHODS AND RATIONALE

The following sections outline the methods and rationale for each of the experiments you will be performing. For each experiment, there is an accompanying set of human disease implications which you should review – these are located further along in the course pack. Experiments are broken up as follows:

OSMOREGULATION:

1) **Human Osmoregulation**
2) **Toad Bladder Water Flux (Part I and Part II)**
3) **Red Cell Water Flux**

Please see the schedule on the first page of this module in order to figure out which experiments to pay particular attention to (not all groups do every experiment).

Osmoregulation

Human Osmoregulation

Many would argue that the best experimental ideas come from observations in our own patients and daily lives. Thus, while the rest of the experiments for the week will use isolated membrane preparations to examine and elucidate properties of human aquaporin analogs and water flux in vitro, throughout the week we will simultaneously be conducting our own "in vivo" experiments. We hope that this portion of the module will not only be fun and educational, but will allow you to use your imagination in designing your own experiments, and generating your own hypotheses.

Using an osmometer, we will measure our own urine osmolalities at various times throughout the day, beginning with the first morning void. We will be recording the urine volume, urine osmolality, and urine chloride concentration for all voids. As chloride is the major urinary anion, it parallels the concentration of the major urinary cations (sodium, ammonium, and potassium).

We would like you to think in advance about potential experiments which could be safely conducted on yourself and your colleagues. The experiments should involve ingestion of either differing osmotic loads for the day (examples include half of the group electing to eat three meals of soup and potato chips, and the other half drinking excess water), or ingestion of substances known or hypothesized to induce natriuresis or diuresis (for example, a common morning drink, or a common weekend drink). Be creative, but safe!

We will meet for a pre-module discussion each morning, and will help you through the hypothesis generation stage. Once we have decided upon the experiments for the day, one person will be in charge of actually recording the urine osmolality, volume, and chloride for all voids throughout the day. At the end of the day, we will review the data and see whether our hypotheses were correct. If correct, we can speculate further about the mechanism, and possibly make suggestions for study for future groups. If incorrect, we can also speculate about why the results came out differently than expected.

Protocol: The osmometer is simple to use and you will receive hands-on instruction in the lab.

Toad bladder osmotic fluxes

Part I

In the following module, we will observe the effects of ADH on the toad bladder first hand, manipulating the concentration gradient between the lumenal and basolateral surfaces. We will then observe the effect of blocking the aquaporin channel on water flux across the bladder; this will be accomplished via addition of mercury, which inhibits water flux through the aquaporin channels. Feel free to design experiments using the toad bladder which simulate the conditions of your simultaneous "human" experiment! Please review the section on "Toad bladder osmotic fluxes" in the *Human Disease Implications* section to gain a better understanding of the human disease correlates which this module is intended to elucidate.

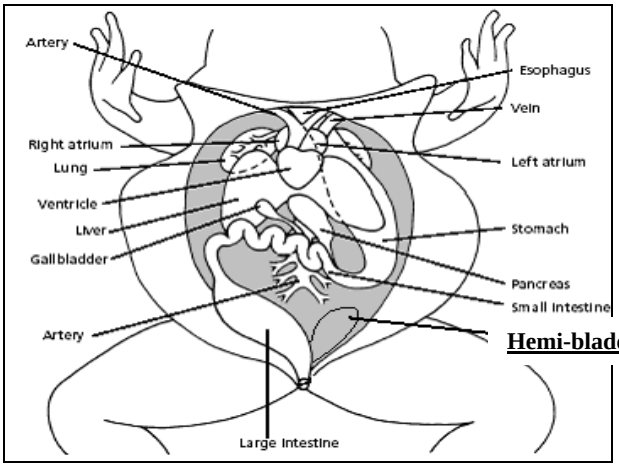
Goals:

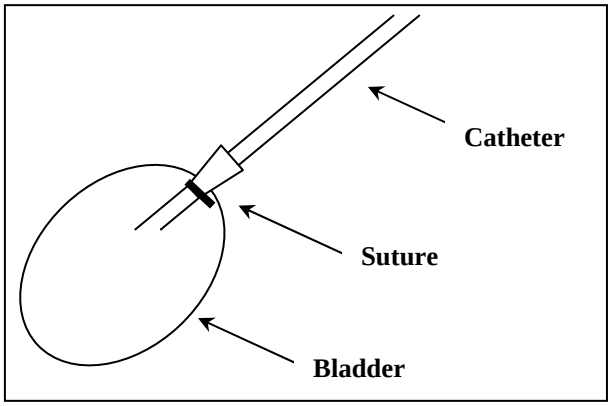
1) To define the effects of ADH on toad urinary bladder water flux.
2) To understand how concentration gradients affect water flux in the absence and presence of ADH.
3) To induce a type of "nephrogenic" diabetes insipidus and observe the effect on water flow.

Protocol:

Isolation of the toad bladder and setup:

1. Open the abdominal cavity and visualize the bladder (see figure to the right).
2. Cut a small hole in the part of the bladder closest to the urethra and insert a catheter into the bladder.
3. Secure a suture around the neck of the catheter, in order to secure the catheter in place inside of the bladder.
4. Dissect out and isolate the bladder, attached to the catheter (see figure below).



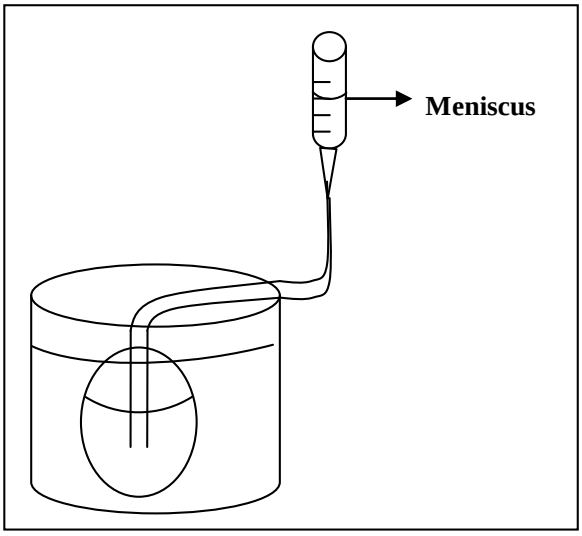


Experiment:

A) Once the bladder attached to catheter are removed from the toad, the bladder will be immersed initially into "toad ringer's solution," an iso-osmotic solution. The remainder of the experiment will involve varying the concentration gradient between the inside and outside of the bladder (apical and basolateral sides, respectively), in the presence and

absence of different hormones/substances. Water flux will be tracked by downward movement of the meniscus in the syringe attached to the catheter which communicates with the fluid inside of the bladder (see figure below). You will record the rate at which the meniscus descends in the presence of different hormones, drugs, and concentration gradients.

B) Fill the hemi-bladder with "toad-ringer's solution." Vary the concentration gradient by varying the solution bathing the basolateral surface of the bladder (options include toad-ringer's solution, 0.45% NS, 0.9% NS, 50% dextrose, etc.), and do this in the presence and absence of vasopressin addition to the basolateral surface. Try adding mercury and observe the effect, knowing what you do about the effect of mercury on aquaporin channels.



Experimental setup

Red cell water fluxes

Whereas the toad bladder osmotic flux focuses on movement of water through inherently water-*impermeable* membranes via insertion of special channels (aquaporins), the following module utilizes water-*permeable* membranes in order to better characterize the mechanisms responsible for general volume shifts within the body. The concept of effective versus ineffective osmols is integral to understanding the movement of water in the body, and the pathophysiology behind certain causes of hyponatremia.

Water moves between the fluid compartments of the body (and into and out of cells) primarily as a result of osmotic forces. A solute that is able to induce movement of water between fluid compartments is termed an "effective" osmol. Inherent in this definition is that the solute itself is limited, in one way or another, to one side of the membrane. An "ineffective" osmol, on the other hand, is one that readily diffuses across membranes, and thus is limited in its ability to incite the movement of water.

Sodium is the most abundant effective osmol – this capacity is derived from the action of the Na/K ATPase pump, which relegates sodium predominantly to the extracellular space, thus holding a corresponding amount of water in the extracellular space. Similarly, potassium is the major intracellular effective osmol, acting to hold water within the cell. In contrast, urea readily crosses cell membranes and thus has roughly equal concentrations in the intracellular and extracellular compartments. Thus, while a rapid change in urea concentration from one compartment to another may lead to an initial water flux, this change would be attenuated over time as urea equilibrates via diffusion from one compartment to another. While it is indeed a measured solute in the extracellular space, and thus contributes to measured extracellular osmolality, it does not effect water flux, and is thus, an ineffective osmol.

In the following module we will observe changes in red blood cell volume in the presence of differing extracellular solutes, in order to better characterize the effectiveness (or ineffectiveness) of those solutes at inducing water flux. Again, please see the section on *Human Disease Implications* to gain a better understanding of the human disease correlates which this module is intended to elucidate.

Goals:

1) To review the concept of effective osmols
2) To understand the difference between tonicity and osmolarity

Protocol: In this experiment we will be utilizing a stopped flow fluorimeter to measure the volume of red blood cells (RBCs) under various different osmotic conditions, and in the presence of various drugs. This device is able to achieve rapid mixing of two solutions loaded into syringes. In this way we can quantitate the effect of a suddenly applied osmotic gradient to membranes of interest. The measurement of water transport in RBCs relies on light scattering in order to quantify volumetric changes; light of a specified wavelength is shone on the RBCs, behind which lies a light-detecting photomultiplier tube; as the RBCs shrink, more light is scattered, leading to an increase in the light hitting the detector. We will start by bathing the RBCs in an iso-osmolar solution (the fluorimeter will accomplish this for you), expecting zero change in volume. Subsequently, we will track rapid volume changes in the RBCs upon mixing with each of the following solutes sequentially to the extracellular environment: Alcohol, ethylene glycol, methanol, mannitol, glycine, sorbitol, glucose, urea, 0.45% NaCl, 0.9% NaCl.

Observe also what happens with the addition of mercury, an antagonist of water movement through the aquaporin channels.

Questions to be answered during the experiments:

What happens to water flux with the addition of each of the above solutes to the extracellular environment? Please use the fluorimeter to prepare sequential plots with time as the “x-axis” and “signal” (the surrogate for RBC volume) as the “y-axis” for addition of the following solutes in the presence and absence of mercury: Alcohol, ethylene glycol, methanol, mannitol, glycine, sorbitol, glucose, urea, 0.45% NaCl, 0.9% NaCl.

Xenopus oocyte water fluxes

In this module, utilizing oocytes of *Xenopus Laevis* (the African Clawed Frog) we will explore the actions of water channels to permit water movement across a normally impermeable membrane. *Xenopus* lay their eggs in pondwater, which has a low osmolality. Prior to your arrival, the oocytes have been injected with RNA encoding a functional aquaporin. The oocyte has proven to be an excellent experimental vehicle for the expression of membrane transporters and ion channels. The oocyte is in effect a protein-synthesizing factory which springs into action upon being supplied with RNA. In these experiments we will be able to study the kinetics of water transport by oocytes which are expressing functional aquaporins. These aquaporins are constitutively inserted into the membrane of the oocytes. Aquaporins transport water bidirectionally; thus, by varying the osmolality of the internal and external environment we will manipulate water flux through the aquaporin molecules and visualize subsequent changes in size of the oocytes, and even rupture of the oocyte membrane under conditions of extreme volume expansion.

Goals:

1) To define the effects of aquaporin expression on membrane water permeability.
2) To understand how concentration gradients affect water flux.
3) To determine whether aquaporins from patients with congenital nephrogenic diabetes insipidus function to move water across the plasma membranes of *Xenopus* oocytes.

Protocol: Using a microscope and microinjector you will inject 10 ng of RNA coding for different aquaporins into the interior of a number of oocytes. You will also assay for aquaporin function by placing oocytes which were injected with RNA several days previously into solutions of varying hypotonicity. A video camera will then record oocyte swelling and the kinetics of this process will be analyzed by image processing in which the cross-sectional area of each oocyte will quantitated by Image J software and the data exported to an Excel spreadsheet.

Toad bladder osmotic fluxes

Part II

In the following module we will build upon the findings of your colleagues from the prior rotation. They will have established the properties of the toad bladder membrane under normal physiologic conditions by varying the concentration gradient between the apical and basolateral surfaces of the hemi-bladder in the presence and absence of vasopressin and mercury (an inhibitor of water flux through the aquaporin channel). Your task today is to observe the physiology in the presence of different substances of your choice. Again, feel free to design experiments using the toad bladder which simulate the conditions of your simultaneous "human" experiment. Just as you did for the human osmoregulation component, you should be able to hypothesize what effect addition your substance to the basolateral membrane of the toad bladder will have.

Goals:

1) To define the effects of various ingested substances on urine osmolality and volume in humans ("in vivo") (this relates to the human osmoregulation component of today's experiments).
2) To use the toad bladder to explore the mechanism of action of these substances ("in vitro").

Protocol:

Isolation of the toad bladder and setup: See previous section.

Once you have determined which substances you will be testing today (3 would be a reasonable target), you will use the same setup for the toad bladder gravimetric analysis as illustrated/described on the previous two pages.

HUMAN DISEASE IMPLICATIONS

Red cell water fluxes

Human disease Implications: Hyperglycemia induced hyponatremia
Glucose acts as an effective osmol in hyperglycemic states by inducing water movement from the intracellular to the extracellular space. Thus, plasma water is increased, resulting in dilutional hyponatremia. For every 100 mg/dL increase in plasma glucose, the plasma sodium concentration will decrease by approximately 2 mEq/L. Interestingly, since plasma osmolarity is actually *increased* in severe hyperglycemia induced hyponatremia, the usual cerebral repercussions of hypo-osmolar hyponatremia (cerebral edema) are not manifest.

Toad bladder osmotic fluxes

Human Disease Implications: Diabetes Insipidus (DI)
Diabetes insipidus (*diabetes means "excess urine," while insipid means "tasteless")* may be either central or nephrogenic. Central DI implies lack of production of ADH by the posterior pituitary from various causes, including Sheehan's Syndrome, pituitary adenoma, genetic syndromes, and infiltrative disorders, to name a few. Nephrogenic DI arises when there is resistance to the action of ADH in the kidney. This can arise secondary to problems with the ADH receptor, or problems with the aquaporin molecule itself, both of which can be caused by genetic mutations, toxic exposures (for example, lithium), or metabolic abnormalities (for example, hypercalcemia).

Questions to ponder:

1) What do you anticipate will happen to water flux across the toad bladder with the addition of mercury?

Human Disease Implications: Syndrome of inappropriate anti-diuretic hormone (SIADH) and hyponatremia
The syndrome of inappropriate anti-diuretic hormone implies the presence of ADH in the spite of one or more conditions making this presence inappropriate. Understanding of the syndrome rests on understanding the normal stimuli for release of ADH, which include both baroreceptor and osmoreceptor functions. ADH is released from the posterior pituitary in response to increased osmolality (the most sensitive stimulus), and decreased effective circulating volume (the most potent stimulus). Thus, in the face of hypovolemia, ADH is elevated, stimulating resorption of water in the collecting duct. This may cause hyponatremia if hypotonic fluids are consumed concurrently (as hypovolemia is the most potent stimulus for ADH release and will be active even in the face of hypo-osmolarity). However, despite the resultant hyponatremia, the ADH release is considered *appropriate* in this setting. If, however, neither of these two stimuli are present (the patient does not have decreased effective circulating volume by exam, and is not hypertonic), and the patient has ADH present (as manifest by a urine osmolarity > 100), this would imply the SIADH.

Questions to ponder:

1) What do you anticipate will happen when ADH is introduced in the presence of a hypo-osmolar solution bathing the outside of the toad bladder?
2) What will be the serum ADH level, serum osmolarity, and urine osmolarity in the following disease states? (Complete the table)

Disease State	**Serum ADH**	**Serum Osmolarity/Serum Na**	**Urine Osmolarity**
Psychogenic Polydipsia			
Central DI			
Nephrogenic DI			
Water deprivation			
SIADH			

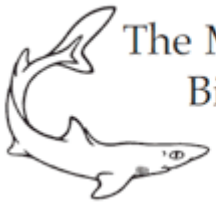
1) What do you anticipate will happen when ADH is introduced in the presence of a hypo-osmolar solution bathing the outside of the toad bladder?
2) What will be the serum ADH level, serum osmolarity, and urine osmolarity in the following disease states? (Complete the table)

Disease State	**Serum ADH**	**Serum Osmolarity/Serum Na**	**Urine Osmolarity**
Psychogenic Polydipsia			
Central DI			
Nephrogenic DI			
Water deprivation			
SIADH			

BIDMC Course on Comparative Physiology

Secretory Module

Beth Israel Deaconess Medical Center



The Mount Desert Island Biological Laboratory

SECRETORY PHYSIOLOGY MODULE

http://www.kynd.net

Jordan Pond and the Bubble, Mt. Desert Island

Goals and Schedule

The goals of the session are focused around understanding mechanisms of secretion as they pertain to ion transport. Students will setup and understand the use of the shark rectal gland as a model of understanding sodium and chloride transport. Each group will build on the work of the last to understand ion transport physiology. A progressive comparison of data derived from each group over the course of the week will help to build the group's understanding of rectal gland secretion and basic pharmacology as it pertains to the $Na^+/K^+/Cl^-$ channel. Using principles of comparative physiology, this model will serve as a tool to understand a variety of clinical problems with ion transport. Clinical correlation discussions along with relevant reading will help students to link lessons learned at the bench to the bedside.

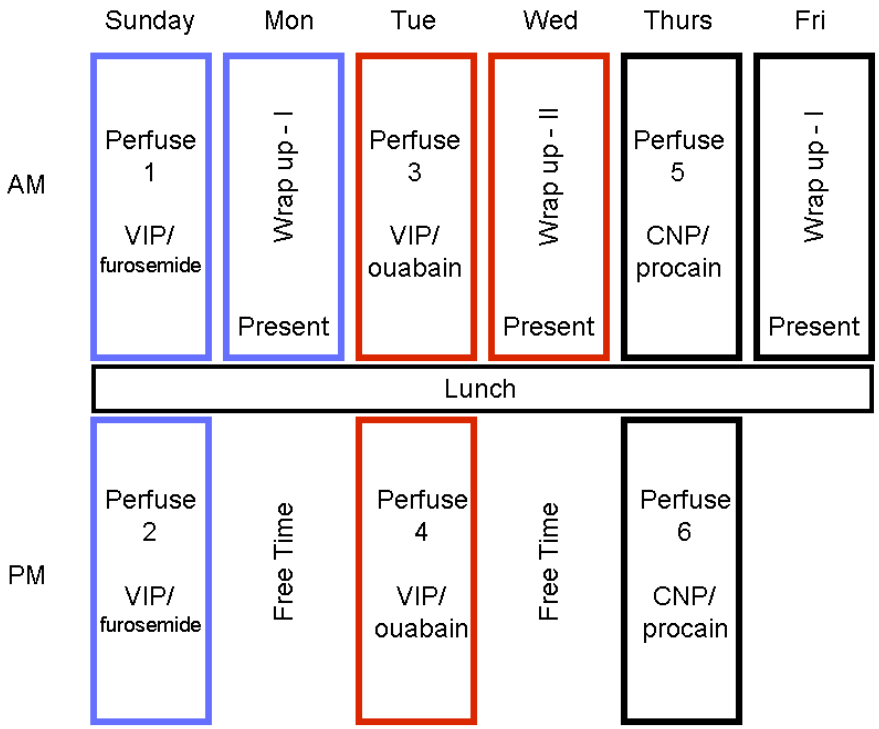
1. To use the shark rectal gland as a model for understanding sodium and chloride transport
2. To quantify the effect of VIP and CNP on rectal gland secretion and what happens with modifying substances
3. To apply this model to understanding the pathophysiology of secretory mammalian disease
4. To gain insight into the effect of key pharmacologic substances on rectal gland secretion

Readings:

Epstein, Franklin H. "The Salt Gland of the Shark." A Laboratory by the Sea. Ed. F.H. Epstein. The River Press, 1998. p. 238-254.

Epstein, F. and Silva, Patricio. "Invited Review: Mechanisms of Rectal Gland Secretion. " *The Bulletin: MDI Biologic Laboratory* 44:1-5 (2005).

Field, M. "Intestinal ion transport and the pathophysiology of diarrhea." J. Clin. Invest. 111:931-934(2003).


Sunday
Mon
Tue
Wed
Thurs
Fri
AM
Perfuse 1 VIP/ furosemide
Wrap up - I Present
Perfuse 3 VIP/ ouabain
Wrap up - II Present
Perfuse 5 CNP/ procain
Wrap up - I Present
Lunch
PM
Perfuse 2 VIP/ furosemide
Free Time
Perfuse 4 VIP/ ouabain
Free Time
Perfuse 6 CNP/ procain

BACKGROUND

http://upload.wikimedia.org/wikipedia/commons/thumb/8/88/Squalus_acanthias2.jpg/800px-Squalus_acanthias2.jpg

Introduction to shark rectal gland

Activators and inhibitors of rectal glad

Intracellular pathways

Please read the first two paper in the references for an introduction to the shark rectal gland and its physiology.

PROTOCOL

In Vitro Perfusion of the Shark Rectal Gland

Materials:
One shark
1-razor blade
1-surgery board
1-pithing wire
3-PE-90 tubing
4-0 silk
Scissors
Kelly clamp
1-microscissors
Several Eppendorf centrifuge tubes
1-sharpie marker

A1: Sacrificing the animal and excision of the rectal gland

1. Use only adult healthy shark (*Squalus acanthias*)
2. Pithing is an acceptable way to sacrifice the animal. Perform this in the following manner:
    a. two people are needed—one to hold the animal straight and the other pith
    b. make a small incision through the cartilage in the nose
    c. insert a straight wire all the way down, almost to the tail of the animal. Try to keep the shark straight
    d. turn the shark on its side
    e. using a pair of scissors open the abdomen starting at the cloaca with the blunt end of the scissors in the shark body (see figure 1)
    f. cut rectal gland free by severing the rectum about 2 cm above the rectal gland and then freeing the gland along the mesenteric attachment
3. Place rectal gland on surgery board pre-wet with shark Ringer's solution

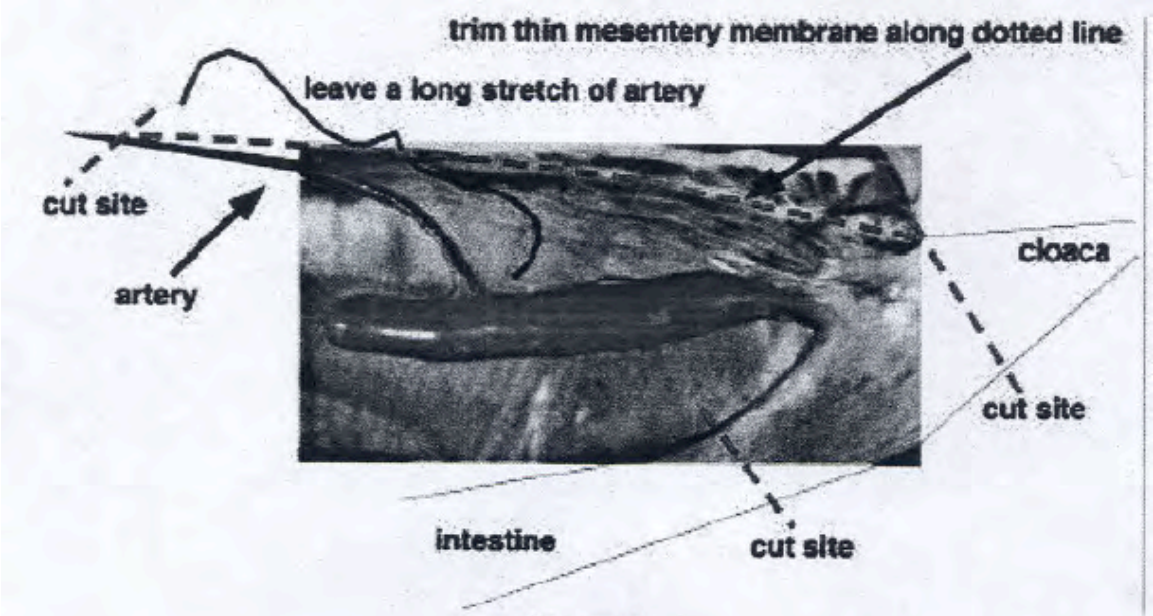


Figure 1.

A2: Setting up the perfusion (see figure 2)

1. clamp the end of the artery with a Kelly clamp to hold the artery firm and taught
2. Cut a piece of PE-90 tubing, 20 cm long, and attach one end to a needle-IV line containing Ringer-glucose perfusate
3. with a razor blade, cut a 45 degree angle out of the other end
4. start a flow of perfusate, and clear the bubbles from the line
5. make a tiny incision in the artery (perpendicular to the artery) with microscissors
6. insert sharp end of the PE-90 tubing into the artery. Look for immediate flow of blood out of the vein.
7. tie the cannula into the vein with 4-0 surgical silk
8. using scissors, open the intestinal segment tube by cutting along the side
9. use forceps to rotate the segment counterclockwise and fold open the flaps to straighten the vein on the underside of the tissue
10. look for the duct opening on the underside of the tissue
11. cut a piece of PE-90 tubing 10-15 cm long with blunt ends
12. using forceps gently open up the duct using gentle pressure
13. insert the PE-90 tubing just a few cm, then fold the left flap of the intestine back over the tubing. Insert the tubing so it can be seen entering the actual duct structure.
14. Observe for secretion. Some glands will quickly produce fluid while others will be much slower. If this is the case, gently stroke the gland to clear it of mucus.

15. Cut piece of PE-90 tubing 10-15 cm long, leaving both ends blunt
16. Insert one end into the vein that same distance as you did for the duct
17. Tie off both tubes using 4-0 silk making sure neither slips out while placing the knot
18. Transfer the gland into a seawater cooling chamber
19. Collect venous effluent into a beaker using a glass bottle and measure it in a graduate cylinder every 10 minutes

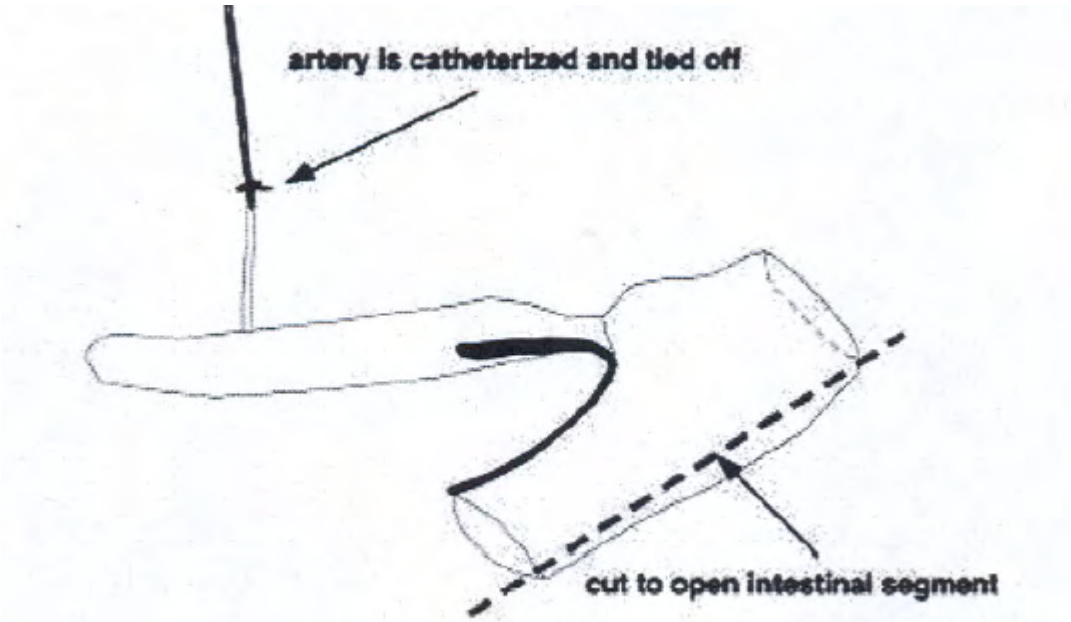


Figure 2

A3: Measuring output of duct

1. Collect fluid directly in preweighed micro tubes. Weigh again to establish volume excreted and record.
2. Change tubes every 10 minutes.

There will be two perfusions set up for each AM and PM session.

A4: Perfusion

1. Start with a control period of perfusion consisting of 2-3 10 min collection in order to establish a basal rate of secretion.
2. A bolus of the compound of interest can then be given over 1 min without altering the rate of perfusion.
3. Continue collection at 10-min intervals until the rate of secretion returns to baseline levels.
4. measure chloride concentration in duct fluid

Questions

All groups:

1. What is the shark rectal gland and what is its function?

2. How does the rectal gland help with its survival? In other words, what si the evolutionary advantage of this for the species?

3. How is NaCl transported across the epithelium lining the rectal gland tubules?

4. What are the cellular signaling pathways that lead to secretion?

Group 1:

How does VIP change rectal gland secretion? How does furosemide change this?

Group 2:

How does ouabain effect rectal gland secretion? Why does it do so?

Group 3:

How does CNP's effect on the rectal gland secretion compare with that of VIP? What occurs in the presence of procaine? What insights does this provide into the mechanism of action of CNP?

Clinical Correlation

Group 1:
What is the role of VIP in mammals and where does it act? What disease states is it associated with?

What is the mechanism of the action of Lasix in humans? How does the effect of Lasix on the shark rectal gland relate to its common clinical use in heart failure?

Group 2:
How is ouabain used in humans? How do you explain its clinical effects?

What other drugs in mammals target Na-K ATPase?

Group 3:

CNP is analogous to BNP in heart failure patients. How would the action of CNP in the shark relate to heart failure in the human?

All groups:

A. What human organs actively transport Cl^- in a way analogous to that used by the rectal gland?

B. **Diarrhea**: The shark rectal gland can serve as a model to understand the mechanisms of secretory diarrhea.

What is the effect of cholera toxin in humans?

How do you use stool chemistries to help evaluate the cause of diarrhea?

What are some potential drug targets for treatment of diarrhea?

C. **Cystic fibrosis**: The CFTR channel is related to chloride secretion in the shark rectal gland. It is under the influence of two pathways.

What is the cellular physiology of the CFTR receptor and how does this relate to the evaluation of cystic fibrosis?

If you were to develop a novel drug for cystic fibrosis, what are some potential drug targets for this disease?